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PRINCIPAL INVESTIGATOR: Jiing-Dwan Lee, Ph.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, California 92037

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Jiing-Dwan Lee, Ph.D.

E-Mail: jdlee@scripps.edu

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Table of Contents

Introduction:

ErbB family of growth factor receptors (ErbB1-4) are critically involved in the derivation of certain mammary cancers [1-3]. Among them, ErbB2/Her2 is most notable as overexpression or amplification of this gene is found in approximately 30% of human breast cancers and people with these ErbB2-overexpressing tumor have a shorter time to relapse and lower overall survival rate than patients whose tumors do not overexpress ErbB2 [4-9]. Since ErbB2 activity plays such an important role in development of breast cancer, it is important to understand the regulatory mechanisms by which mammary cells maintain the proper intensity and specificity of ErbB2-generated signaling. ErbB receptors promote tumor progression by sending oncogenic signals into cells through its cytoplasmic signaling domain [10-14]. In this regard, we discovered that the signaling domain of ErbB2 interacts with Tid1 protein and that the increased expression of this protein in ErbB2-overexpressing breast cancer cells promotes ubiquitinization and proteosomal degradation of ErbB2 resulting in potent inhibition of ErbB2-dependent intracellular signaling. We hypothesize that Tid1 act as a tumor suppressor in breast tumorigenesis associated with ErbB2 malfunction. The proposed study should verify our hypothesis and test the therapeutic potential of Tid1 in treating tumor overexpressing ErbB2. To investigate the function and molecular action of Tid1 gene as a tumor suppressor in the development of breast cancer, we propose (1) to investigate Tid1's role in the onset and progression of breast tumor using in vivo animal model (2) Further explore the functional role of Tid1 on both signaling events and functional responses mediated by ErbB2 in breast cancer cells (3) identify structural domains in Tid1 required for recognition and inhibition of ErbB2 signaling.

Body:

Task 1. To investigate Tid1's role in the onset and progression of breast tumor using in vivo animal model.

1.1. Breeding for mice with genotypes ($Tid1^{flox/flox}$, MMTV-neu/MMTV-neu, MMTV-Cre/+) ($Tid1^{flox/flox}$, MMTV-Cre/+) ($Tid1^{flox/flox}$, Wap-Cre/+) and their littermate controls. (1-18 months)

1.2. Analyzing and characterizing tumorigenesis of the mice generated above. (13-36 months)

Progress: We have obtained $Tid1^{flox/+}$, MMTV-Cre mice (mix genetic background) and are now backcrossing these mice (for the fifth times) with FVB/n mice to generate floxed Tid1 and MMTV-Cre alleles in FVB/n background. We plan to analyze mammary tumorigenesis in these mice after 8-10 backcrossing.

Task 2. To further explore the functional role of Tid1 on both signaling events and functional responses mediated by ErbB2 in breast cancer cells

1.1. Study the scope of the inhibitory effect of Tid1 on ErbB2-dependent intracellular signal transduction (6-24 months).

1.2. Examine the biological effect of Tid1 on the malignant properties of breast cancer cells (13- 30months).

Progress: We discovered that (1) increased cellular Tid1 inhibit the activities of oncogenic signaling pathways, Erk1/2 and BMK1/Erk5, in mammary carcinoma cells (Figure 6 & 7 in accompanying publication #1 [Cancer Research 64: 7732-7739, 2004]). (2) Tid1 inhibits the malignant growth and induced apoptosis of Her2/ErbB2-overexpressing cancer cells (Figure 3 & 4 in accompanying publication #1). (3) Tid1 facilitates the ubiquination and degradation of Her2/ErbB2 in cancer cells (Figure 6 in accompanying publication #1) (4) Tid1 inhibits the growth of ErbB2-dependent tumor in animal (Figure 8 in accompanying publication #1).

Task 3. To identify structural domain(s) in Tid1 required for recognition and inhibition of ErbB2 signaling.

2.1. Generating Tid1 deletion mutants and mutants with specific function domain destroyed. Also produce recombinant adenovirus encoding these mutants to infect various breast cancer cells for the functional test proposed in aim 3 (13-24 months).

2.2. Identification of the minimal structural domains of Tid1, which contribute to the binding of ErbB2 and the motif in Tid1 required for blocking ErbB2 onocogenic signaling (19-36 months).

Progress: We generated Tid1 deletion mutants and mutants with specific function domain destroyed (Figure 1 in accompanying publication #1) as well as recombinant adenoviruses encoding these mutants (Figure 3 & 4 in accompanying publication #1). We demonstrated that C-terminal portion and DnaJ domain of Tid1 are required for its inhibitory effect on Her2/ErbB2 signaling (Figure 4 & 5 in accompanying publication #1).

Key Research accomplishments:

- Co-localization of ErbB2 and Tid1 in mammary carcinoma cells.
- Increased cellular Tid1 induces growth arrest and cell death in ErbB2 overexpressing breast cancer cells.
- Increased cellular Tid1 leads to apoptosis of cancer cells. (A) Diagram of Tid1 deletion mutant constructs.
- The DnaJ and C-terminal domains of Tid1 are critial for the Tid1-mediated PCD in breast cancer cells overexpressing ErbB2.
- $Tid1_S$ negatively regulates ErbB2 signaling pathways by enhancing the degradation of ErbB2.

- Downregulation of Erk1/2 and BMK1 MAP kinase pathways contribute to the Tid1-mediated PCD of mammary cancer cells overexpressing ErbB2.
- Increased cellular Tid1 inhibits the growth of ErbB2-dependent tumors in animals.
- Reducing the expression of Tid1 in breast cancer cells enhanced their migration without affecting their survival or growth rate (accompanying publication #2).
- From microarray screening, we discovered that after Tid1-depletion the mRNA level of interleukin-8 (IL8) was significantly increased in these cancer cells, which consequently increased secretion of IL8 protein by 3.5 fold (accompanying publication #2).
- The enhanced migration of these Tid1-knockdown cells was blocked by reducing the IL8 expression or by adding an IL8 neutralizing antibody to the culture media, suggesting that enhancement of cell motility in these Tid1-deficient cells is dependent on the *de novo* synthesis of IL8 (accompanying publication #2).
- We found that abrogating the NF-κB binding site in the IL8 promoter completely blocked the Tid1 depletion-induced IL8 expression in the breast cancer cells (accompanying publication #2).
- As increased IL8 levels are known to promote tumor metastasis, we tested the effect of Tid1 knockdown on tumor metastasis and found that Tid1 depletion enhanced the metastasis of breast cancer cells in animals (accompanying publication #2).
- Tid1 negatively regulates the motility and metastasis of breast cancer cells, mostly likely through attenuation of NF-κB activity on the promoter of the *IL8* gene (accompanying publication #2).

Reportable Outcomes:

1. Kim, S. W., Chao, T.-H., Xiang, R., Lo, J.-F., Campbell, M. J., Fearns, C. and **Lee, J. D.**, Tid1, the human homologure of a Drosophila tumor suppressor, reduces the malignant activity of erbB-2 incarcinoma cells. *Cancer Research* 64, November 1st, 64(21): 7732, 2004.

2. Kim, S. W., M. Hayashi, J. F. Lo, C. Fearns, R. Xiang, G. Lazennec, Y. Yang, and **J. D. Lee.** Tid1 negatively regulates the migratory potential of cancer cells by inhibiting the production of interleukin-8. *Cancer Res.* 65:8784-8791, 2005.

Conclusions:

Tid1 is the human counterpart of *Drosophila* tumor suppressor Tid56. Tid56 null mutation causes tumorous imaginal discs resulting from continuous cell proliferation without differentiation. To date, the mechanism of tumor suppression of Tid56 in *Drosophila*, as well as the cellular function of Tid1 in human tumorigenesis, are poorly understood. To this end, we discovered that the signaling domain of ErbB2/Her2/neu interacts with Tid1 protein and that the increased expression of this protein in ErbB2-

overexpressing breast cancer cells promotes ubiquitination and proteosomal degradation of ErbB2 resulting in potent inhibition of ErbB2-dependent intracellular signaling and proliferation/survival of these cancer cells (accompanying publication #1). Moreover, by depleting the physiological levels of Tid1 in breast cancer cells using the technique of RNA interference (RNAi), we discovered that the metastastic potential of Tid1-knockdown cells was substantially enhanced due to the increased expression of interleukin-8 (IL-8) resulting from enhanced NF-κB activity (accompanying publication #2). Thus, from these abovementioned results, we suspect that since Tid1 attenuates cancerous signals generated from the ErbB2 receptor as well as negatively regulates the metastatic potential of breast cancer cells by inhibiting the activity of NF-κB and consequently decreasing the expression of IL-8, Tid1, like its *Drosophila* counterpart, may be an important tumor suppressor, especially during mammary tumorigenesis. This grant support has facilitated the understanding of the mechanistic role of Tid1 protein, as a tumor suppressor, in the tumorigenesis and metastasis of breast cancer both at the molecular/cellular level and in an organismal context. Since patients with ErbB2-overexpressing or higher metastatic potential tumors have a considerably worse prognosis and are often unresponsive to conventional treatment, the experimental results described herein should help in developing more effective and safer strategies for inhibiting the growth and spread of this type of aggressive breast tumor.

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Appendices:

1. Kim, S. W., Chao, T.-H., Xiang, R., Lo, J.-F., Campbell, M. J., Fearns, C. and **Lee, J. D.**, Tid1, the human homologure of a Drosophila tumor suppressor, reduces the malignant activity of erbB-2 incarcinoma cells. *Cancer Research* 64, November 1st, 64(21): 7732, 2004.

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[CANCER RESEARCH 64, 7732–7739, November 1, 2004]

Tid1, the Human Homologue of a *Drosophila* Tumor Suppressor, Reduces the Malignant Activity of ErbB-2 in Carcinoma Cells

Sung-Woo Kim,[1] Ta-Hsiang Chao,[1] Rong Xiang,[1] Jeng-Fan Lo,[1] Michael J. Campbell,[2] Colleen Fearns,[1] and Jiing-Dwan Lee[1]

[1]Department of Immunology, The Scripps Research Institute, La Jolla, California; and [2]Department of Surgery, University of California, San Francisco, California

ABSTRACT

The ErbB-2/HER-2 receptor tyrosine kinase is overexpressed in a wide range of solid human tumors. The ErbB-2 gene product is a transmembrane glycoprotein belonging to the epidermal growth factor receptor family, and its cytoplasmic domain is responsible for sending the mitogenic signals into cells. We discovered that this domain of ErbB-2 interacts with Tid1 protein, the human counterpart of the *Drosophila* tumor suppressor Tid56, whose null mutation causes lethal tumorigenesis during the larval stage. Tid1 also is known as a cochaperone of heat shock protein 70 (HSP70) and binds to HSP70 through its conserved DnaJ domain. We found that increased expression of Tid1 in human mammary carcinomas overexpressing ErbB-2 suppresses the expression level of ErbB-2 and attenuates the resultant ErbB-2–dependent oncogenic extracellular signal-regulated kinase 1/2 and big mitogen-activated protein kinase 1 signaling pathways leading to programmed cell death (PCD). A functional DnaJ domain of Tid1 also is required for its inhibition of ErbB-2 expression and the consequent PCD of carcinoma cells resulting from increased Tid1 expression. Importantly, ErbB-2–dependent tumor progression in animals is inhibited by increased expression of Tid1 in tumor cells. Collectively, these results suggest that Tid1 modulates the uncontrolled proliferation of ErbB-2–overexpressing carcinoma cells by reducing ErbB-2 expression and as a result suppresses the ErbB-2–dependent cancerous signaling and tumor progression. Moreover, the cochaperonic and regulatory functions of Tid1 on HSP70 most likely play an essential role in this antitumor function of Tid1 in carcinoma cells.

INTRODUCTION

ErbB-2 is a receptor tyrosine kinase that heterodimerizes with other members of the ErbB family. Its active cytoplasmic domain provides docking sites for various signaling molecules that link ErbB-2 activation to numerous intracellular signaling pathways leading to a variety of cell responses, including proliferation, differentiation, survival, and apoptosis (1, 2). Deregulation of the ErbB-2–dependent signaling network by ErbB-2 malfunction is implicated in the development of malignancy in numerous types of human cancers (3). Overexpression of ErbB-2 is found in nearly 30% of human breast and ovarian cancers and is associated with an unfavorable prognosis (4). Therefore, identifying molecules that interact with the intracellular domain of ErbB-2 will contribute not only to the elucidation of the regulatory mechanisms of ErbB-2 activity in cancer progression but also to the development of new treatments to control the growth of human cancers.

To this end, by using the cytoplasmic region of ErbB-2 as bait in a yeast two-hybrid screening, we discovered that the signaling domain of ErbB-2 interacts with Tid1 protein, which is the human counterpart of the *Drosophila* tumor suppressor Tid56 (5). Tid56 null mutation causes tumorous imaginal discs resulting from continuous cell proliferation without differentiation (5). To date, the mechanism of tumor suppression of Tid56 in *Drosophila* and the cellular function of Tid1 in human tumorigenesis are poorly understood. After confirming that Tid1 interacts with ErbB-2 in mammalian cells, we found that increasing the expression of this protein in breast cancer cells has a potent inhibitory effect on ErbB-2–dependent intracellular signaling and on the subsequent proliferation of breast cancer cells. These results suggest that Tid1 attenuates signals generated from the ErbB-2 receptor and, like its *Drosophila* counterpart, may be an important tumor suppressor, especially in breast cancer.

MATERIALS AND METHODS

Reagent and Antibodies. Unless stated otherwise, all of the other reagents were purchased from Sigma (St. Louis, MO), and all of the antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antiphospho–extracellular signal-regulated kinase (ERK) 1 or phosphor ErbB-2 antibodies were purchased from Upstate (Lake Placid, NY). Maria Rozakis-Adcok (McMaster University, Hamilton, ON, Canada) provided anti-Tid1 immunoblotting antibody. H5509 antibody, Tid1 COOH-terminus–specific antibody, was generated by immunizing rabbits with the KLH (Pierce, Rockford, IL)-conjugated peptide (PGTQTDQKIRMGGKGIPRINS*C*). The italicized Cys residue was added at the COOH-terminal end of each peptide for KLH conjugation reaction. M6889, Tid1 antibody described previously (6), was used for Tid1 immunoprecipitation.

Yeast Two-Hybrid Screening. The cytoplasmic tail (amino acids 676 to 1255) carrying the nucleotide binding site mutation (K753A) of human ErbB-2 cDNA was cloned into the *Nco*I and *Sal*I sites of pGBKT7 (Clontech, Palo Alto, CA) to serve as a bait vector. A human carcinoma cDNA library prepared in the pGAD-GH vector (Clontech) was used as the prey library. The mutant forms of hTid1 encoding Δ168, Δ235, ΔCT, CT, or ΔNT were fused in-frame with the GAL4-DNA activating domain of the pGAD-GH vector to define the region of hTid1 interacting with ErbB-2.

Tumor Cell Cultures. MCNeuA was derived from neu-overexpressing spontaneous mammary tumors that developed in female murine mammary tumor virus/*neu* transgenic mouse with FVB/NJ genetic background (7).

Recombinant Adenoviruses, Infections, and Transfection. A full-length cDNA encoding human Tid1 or its mutants was PCR amplified and cloned into the *Bgl*II and *Hin*dIII sites of pAdTrack-cytomegalovirus adenovirus shuttle vector (Q-BIOgene, Carlsbad, CA). Adenoviruses containing Tid1 or its mutant were used to infect breast cancer cell lines in 24-well plates (1×10^4/well) for 6 hours. Thereafter, the growth media were replaced every 2 days until completion of the experiment. Infection efficiency was checked by green fluorescence at a multiplicity of infection (1×10^7 to 3×10^7 VP). The adenovirus-infected cells were transfected with 1 μg/mL of expression plasmids encoding MEK1E or MEK5D (8) or both, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Apoptosis and Survival Assay. The MTT assay was used to analyze the survival of the cells. Briefly, 1×10^4 cells were seeded in each well of a 24-well plate. Ten microliters of MTT solution were added to cells and incubated at 37°C for 3 hours. The supernatant was removed, and 200 μL of DMSO were added directly to the cells. The MTT color reaction was analyzed using a microplate reader set at $A_{560\,nm}$. For apoptosis assay, apoptotic nuclear changes were detected with an *in situ* cell death detection kit, TMR red (Roche, Basel, Switzerland). The nuclei of cells were counterstained with 4′,6-diamidino-2-phenylindole staining dye (Molecular Probes Inc., Eugene, OR) for

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20 minutes. For internucleosomal DNA cleavage assays, DNA preparation and agarose electrophoresis were performed as described previously (6).

Immunofluorescence. Tumor cells were plated overnight on collagen-coated glass coverslips in 12-well plates (Fisher Scientific, Hampton, NH), fixed, and permeabilized as described previously (9). After blocking in 2% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 hour, the cells were incubated with antibody Neu C-18 (Santa Cruz Biotechnology, Inc.) to detect ErbB-2 or with RS-11 (Santa Cruz Biotechnology, Inc.) to detect Tid1 at 1:100 dilution for 1 hour. To amplify the Tid1 signal, the cells were incubated with biotinylated goat antimouse IgG (Vector Laboratories) for 30 minutes. The cells subsequently were incubated with Alexa Fluor 568 goat-antirabbit (red-orange) or streptavidin-conjugated–Alexa Fluor 488 antimouse antibody (green; Molecular Probes, Inc.) for 30 minutes at 1:1000 dilution. The cells were viewed and photographed with a Bio-Rad MRC1024 laser scanning confocal microscope (Hercules, CA) attached to a Zeiss Axiovert S100TV microscope (Oberkochen, Germany).

Syngenic Tumor Production. Before implantation, the tumor cells were washed twice with PBS, counted, and resuspended in PBS. MCNeuA cells (1×10^6 cells in 100 μL PBS) were inoculated subcutaneously into the right flank of FVB/NJ mice. Tumors were allowed to develop to a size of 30 mm^3. Adenovirus containing $Tid1_S$ (5×10^9 particles of virus in 100 μL PBS) were administered intratumorally at days 8, 12, and 14 after inoculation. Tumor volume (V) was calculated by: $V = (L \times W^2)/(\pi/2)$ for each tumor, where L is tumor length, and W is tumor width, and was measured at various time points as indicated after inoculation.

RESULTS

The cytoplasmic domain of ErbB-2 was used as bait in a yeast two-hybrid screen. A total of 3×10^7 transformants were screened from a human carcinoma cDNA library, and 67 positive clones were selected for sequence analysis based on their potential interaction with the intracellular part of ErbB-2. Using the BLAST algorithm and the nucleotide database at the National Library of Medicine, three clones were found to encode the COOH-terminal portion of $Tid1_S$ protein. As shown in Fig. 1*A* and *B*, the growth of transformed yeast in media lacking His and Ade is completely dependent on the presence of sequences from $Tid1_S$ and ErbB-2 in this GAL4 system. Two splicing isoforms of Tid1 exist in human cells: the long form, $Tid1_L$, and the short form, $Tid1_S$ (10). Tid1 protein contains several distinct domains: an NH_2-terminal signal sequence, a J-domain, a cysteine-rich domain resembling zinc finger repeat (CXXCXGXG), and a nonhomologous COOH-terminal domain (10). To determine which domain of Tid1 was responsible for binding to the ErbB-2 cytoplasmic domain, we tested the affinity between the signaling domain of ErbB-2 and various regions of $Tid1_S$ by yeast two-hybrid–based assays. We discovered that the COOH-terminus (amino acids 292 to 453) of $Tid1_S$ is required and sufficient for the interaction of Tid1 with ErbB-2 (Fig. 1*B*). Using the yeast expression vector encoding AD/$Tid1_L$ fusion protein in the yeast two-hybrid assay described previously, we showed that $Tid1_L$, the splicing variant of $Tid1_S$, also interacts with the signaling domain of ErbB-2 with similar affinity to that between ErbB-2 and $Tid1_S$ (Fig. 1*B*).

To further study whether ErbB-2 and Tid1 exist endogenously as a complex in mammalian cells, cell extracts from SK-BR-3 cells were immunoprecipitated with anti-Tid1 or control antibodies. The immunocomplexes were analyzed by immunoblot analysis with anti–ErbB-2 or antiphospho–ErbB-2 (pErbB-2) antibodies. As shown in Fig. 1*C*, ErbB-2 and the phosphorylated ErbB-2 copurified with Tid1. It is known that Tid1 interacts with heat shock protein 70 (HSP70) through its DnaJ domain. Therefore, it is likely that some of the cellular ErbB-2 may complex with HSP70 because of the interaction between the Tid1/ErbB-2 complex and HSP70 through the DnaJ domain of Tid1. To test this, anti-Tid1 or anti–ErbB-2 antibodies were used separately to immunoprecipitate Tid1 or ErbB-2 in the cell lysates of SK-BR-3 cells, followed by immunoblot analysis with anti-HSP70 antibody or anti-Tid1 antibody. As predicted, we found that HSP70 protein of SK-BR-3 cells exists in the immunocomplexes derived from either anti-Tid1 or anti–ErbB-2 antibodies (Fig. 1*D*). Furthermore, the short form and the long form of Tid1 were detected in the immunoprecipitates of either anti-HSP70 or anti–ErbB-2 (Fig. 1*D*). These results indicate that both forms of Tid1 interact with HSP70 and with ErbB-2.

We next investigated whether endogenous Tid1 could colocalize with the cytoplasmic domain of ErbB-2. ErbB-2 and Tid1 in SK-BR-3 cells were labeled with fluorescence by immunostaining, and their subcellular localization was visualized by confocal microscopy (Fig. 2). In SK-BR-3 cells, Tid1 is dispersed throughout the cytosol and partially colocalized with ErbB-2 (yellow, indicated with arrows, Fig. 2, *top*). This partial ErbB-2/Tid1 colocalization was blocked by overexpression of $Tid1_S$ COOH-terminal mutant (CT), which contains the

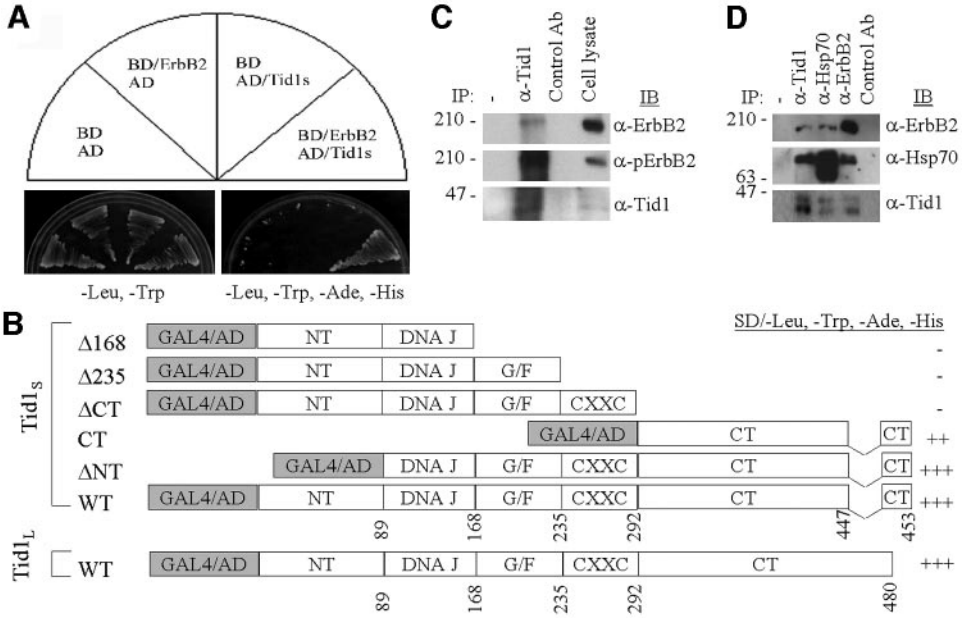

Fig. 1. Tid1 physically interacts with the signaling domain of ErbB-2. *A.,* The yeast strain PJ69–2A was cotransformed with vectors encoding a GAL4 DNA binding domain (*BD*) fusion protein (BD/cytoplasmic domain of ErbB-2) along with the indicated GAL4 active domain (*AD*) fusion protein. Transformed yeast was cultured on plates containing the indicated selective media. *B.* The COOH-terminus of $Tid1_S$ is required for interaction with ErbB-2. Schematic representations of Tid1 protein and its deletion derivatives used as bait in the yeast two-hybrid screen. The presence (+) and absence (−) of interaction between Tid1 mutants and ErbB-2 is shown. *C.* Lysates from SK-BR-3 were immunoprecipitated with either M6689, an anti-Tid1 antibody (6), or control antibodies as indicated. Immunocomplexes were electroblotted onto nitrocellulose filters, and coprecipitated ErbB-2, pErbB-2, or precipitated Tid1 was detected with anti–ErbB-2, anti–pErbB-2, or anti-Tid1 antibodies separately. In total cell lysates, the presence of endogenous Tid1 and ErbB-2 proteins was detected with either anti-Tid1 or anti–ErbB-2 antibodies (last lane of each panel). *D.* The immunoprecipitated samples from SK-BR-3 cells using anti-Tid1, anti–ErbB-2, anti-HSP70, or control antibodies were immunoblotted with anti-HSP70 or anti-Tid1 antibodies.

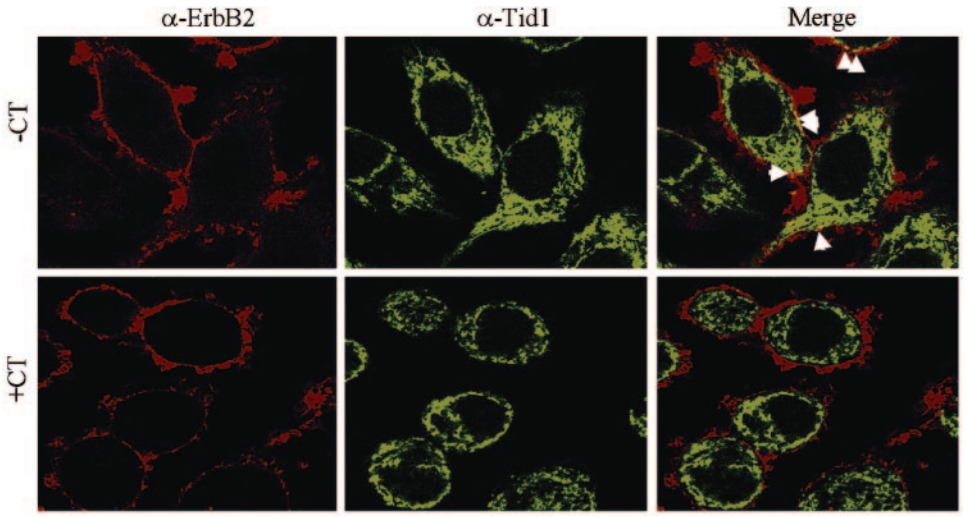

Fig. 2. Colocalization of ErbB-2 and Tid1 in mammary carcinoma cells. SK-BR-3 cells were infected with (*bottom*) or without (*top*) COOH-terminal mutant form of Ad-Tid1$_S$ and were immunostained with anti–ErbB-2 and anti-Tid1 antibodies to detect the colocalization of ErbB-2 (*left, red fluorescence*) and Tid1$_S$ (*middle, green fluorescence*). The merge of red and green fluorescence is shown in the right panels (*yellow*). Colocalization is indicated with arrows.

domain of Tid1 that interacts with ErbB-2 (Fig. 2, *bottom*). The colocalization of ErbB-2 with hTid1$_S$ appears to occur near the inner plasma membrane, where the ErbB-2 cytoplasmic tail most likely is located. As expected, in previous reports it was shown that Tid1 is distributed in the cytoplasm and the nucleus because it interacts with several cellular proteins (10–12). Moreover, in *Drosophila*, Tid56 was reported to interact with the Hedgehog-bound Patched receptor (13). Our data of the interaction between Tid1 and ErbB-2 and the colocalization of Tid1 and ErbB-2 further support the notion that Tid1 interacts with membrane-bound receptors not only in insect cells but also in mammalian cells.

To assess the function of Tid1 in human breast cancer cell lines, breast carcinoma cells with high expression levels (SK-BR-3 and BT-474) or with low expression levels (MDA-MB-231) of ErbB-2 were tested (Fig. 3*A*). Cells were infected with recombinant adenovirus encoding either Tid1$_S$ or Tid1$_L$ resulting in increased levels of Tid1$_S$ or Tid1$_L$, respectively (Fig. 3*B*). Proliferation of ErbB-2–overexpressing cells infected with Ad-Tid1$_S$ was inhibited after 48 hours, and the number of viable cells later gradually decreased, most likely because of cell death (Fig. 3*C*). Increased expression of Tid1$_L$ had a similar effect to that of Tid1$_S$ in these cell lines (Fig. 3*C*). Interestingly, the growth-inhibitory effect of overexpression of Tid1$_L$ or Tid1$_S$ was not observed in MDA-MB231 cells, which express only low levels of ErbB-2. These data indicate that increased Tid1 expression blocks the proliferation of human carcinomas that overexpress ErbB-2.

To ascertain whether the growth inhibitory and cell death effect of Tid1$_S$ in ErbB-2–overexpressing cells is caused by apoptosis and to locate a domain(s) of Tid1$_S$ responsible for the cell death, we generated recombinant adenovirus encoding wild-type or mutant derivatives of Tid1$_S$ proteins (Fig. 4*A*). SK-BR-3 cells were infected with these viruses and checked for expression of the wild-type or mutant Tid1 proteins (Fig. 4*B, top*). Typical apoptotic morphology characterized by loss of adherence, condensed cytoplasm, and formation of

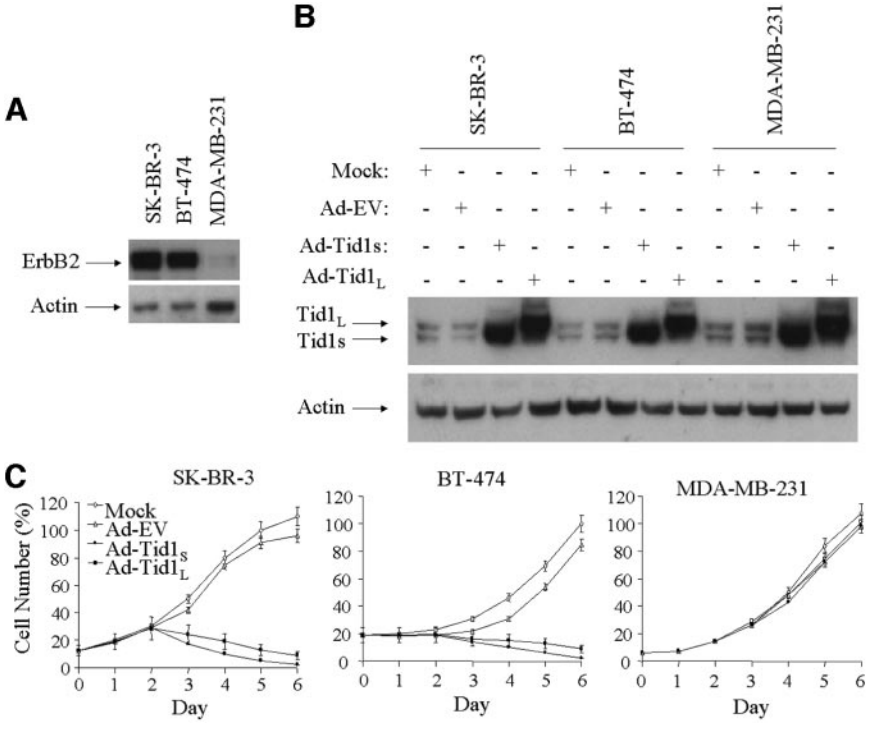

Fig. 3. Increased cellular Tid1 induces growth arrest and cell death in ErbB-2–overexpressing breast cancer cells. *A.* The levels of ErbB-2 expression were detected in the cell lysates of SK-BR-3, BT-474, and MDA-MB-231 with an anti–ErbB-2 antibody in an immunoblot assay. *B,* overexpression of Tid1$_L$ or Tid1$_S$ in various cancer cell lines. Cells were mock infected or were infected with empty virus (Ad-EV) or with viruses encoding either Tid1 short form (Ad-Tid1$_S$) or long form (Ad-Tid1$_L$) as indicated. Two days after infection, levels of Tid1 were analyzed by immunoblot analysis. *C.* SK-BR-3, BT-474, and MDA-MB-231 cells were treated as in *B*. The growth of these cells was analyzed by MTT assay at the indicated day after infection. The MTT value of the day 6 mock-infected cells was taken as 100%.

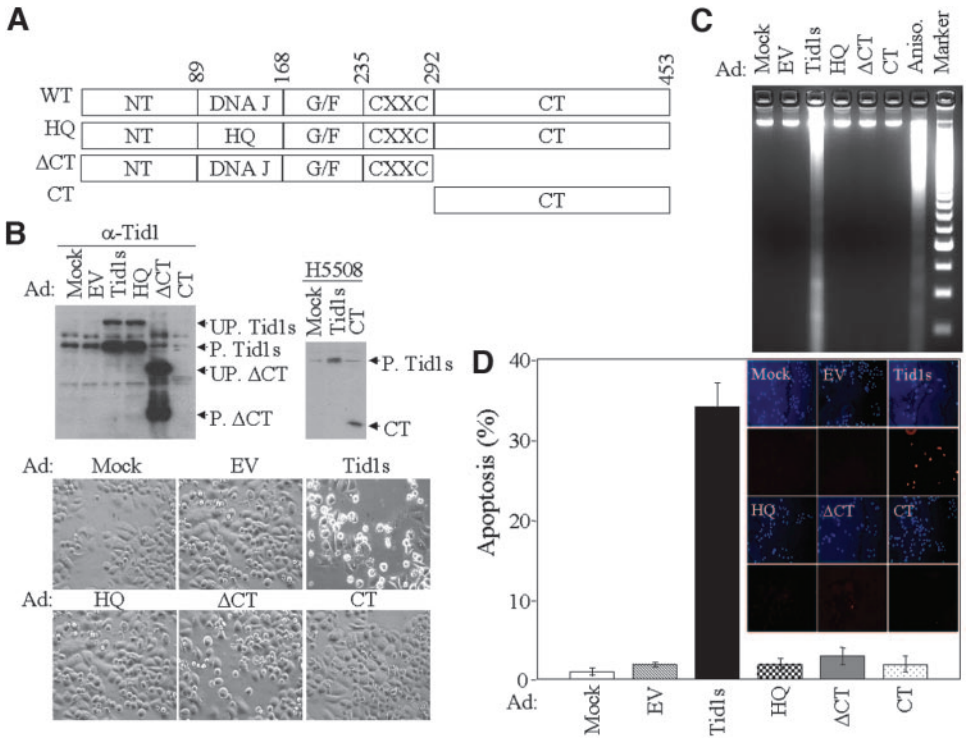

Fig. 4. Increased cellular Tid1 leads to apoptosis of cancer cells. *A,* diagram of Tid1 deletion mutant constructs. *B,* SK-BR-3 cells were mock infected (*Lane 1*) or infected with Ad-EV (*Lane 2*), Ad-Tid1$_S$ (*Lane 3*), or its mutants as indicated (*Lanes 4* to *6*). Cell extracts from these cells were analyzed by immunoblot analysis using anti-Tid1 antibody (*top left*). Because this anti-Tid1 antibody is not able to detect the COOH-terminal of Tid1$_S$, H5509, an antibody that specifically detects the COOH-terminus of Tid1, was used for detecting the expression of Tid1$_S$-CT mutant (*top right*). The arrows indicate the processed form (*P*) or the unprocessed form (*UP*) of Tid1$_S$ and its mutants. *Bottom,* phase contrast images of cells 6 days after infection with the indicated viruses. *C,* SK-BR-3 cells were infected as in *B*. Seventy-two hours after infection, genomic DNA from these cells was prepared and analyzed for DNA laddering. As a positive control for DNA laddering, cells were treated with 5 μg of anisomycin/mL for 18 hours. *D,* SK-BR-3 cells were treated with viruses as in *B*. Six days after infection, these cells were stained with 4′,6-diamidino-2-phenylindole (blue) and the *in situ* TUNEL assay (*red*; Cell Death Detection Kit, TMR red); *% apoptosis*, red cell number per total cell number.

apoptotic bodies in dying cells was observed by light microscopy 6 days postinfection with Ad-Tid1$_S$ (Fig. 4*B, bottom*). Similar results also were found in the cells infected with Ad-Tid1$_L$, indicating Tid1$_L$ has a similar apoptosis-inducing effect on carcinoma cells as Tid1$_S$ (data not shown). The appearance of a DNA ladder indicative of typical apoptosis was detected in samples prepared from cells infected with Ad-Tid1$_S$ 3 days after infection (Fig. 4*C*). In contrast, no apoptosis or DNA laddering was observed in cells infected with the Ad-Tid1$_S$ mutants (Fig. 4*B, bottom* and *C*). The percentage of cells undergoing programmed cell death (PCD) was quantified by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay (Fig. 4*D*). Approximately 35% of the ErbB-2–overexpressing cells with increased expression of Tid1$_S$ were apoptotic, whereas apoptotic bodies were found in only 1% to 3% of those cells infected with control adenovirus or adenoviruses encoding mutant Tid1$_S$. Interestingly, none of the Tid1$_S$ mutants, including the J domain mutant (HQ), COOH-terminal deletion mutant (ΔCT), and CT mutants (Fig. 4*A*), induced apoptosis. These data suggest that the DnaJ domain (critical for the binding of Tid1 to HSP70) and the COOH-terminus of Tid1 (responsible for its interaction with ErbB-2; Fig. 1*B*) are required for Tid1-mediated apoptosis. Furthermore, active caspase-3 increased significantly in the cells infected with Ad-Tid1$_S$ compared with those cells uninfected (mock) or infected with control virus (Ad-EV), confirming that the apoptosis induced by increased intracellular Tid1 is concomitant with the production of active caspase-3 (Fig. 5*A*). Treatment with z-Val-Ala-Asp, a caspase-3 inhibitor, also was able to attenuate cell death induced by increased expression of Tid1 in SK-BR-3 cells (Fig. 5*B*), indicating that caspase-3 is involved in this Tid1-induced apoptosis. To further examine the contribution of each domain of Tid1 in Tid1-mediated cell death, we tested whether apoptosis induced by increased Tid1 can be influenced by intracellular expression of Tid1 mutants. Ad-Tid1$_S$–

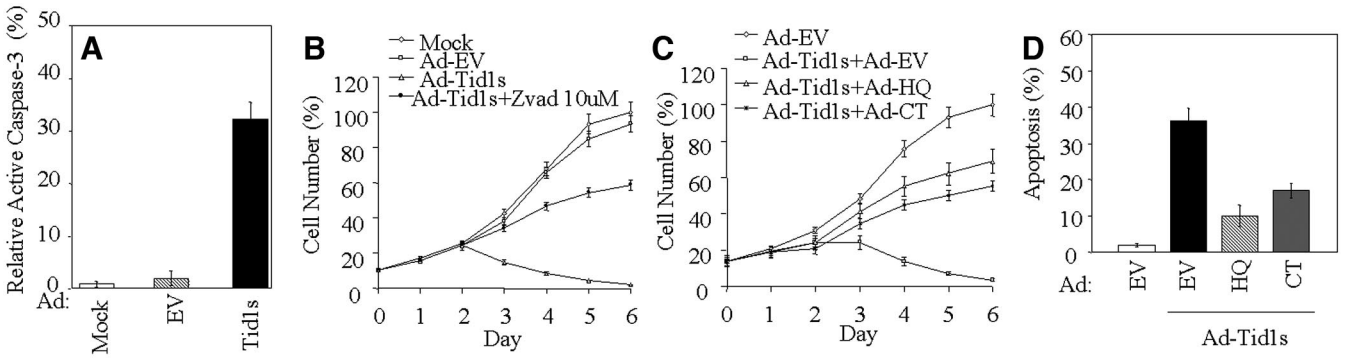

Fig. 5. The DnaJ and COOH-terminal domains of Tid1 are critical for the Tid1-mediated PCD in breast cancer cells overexpressing ErbB-2. *A,* SK-BR-3 cells were mock infected or infected with control virus (Ad-EV) or Ad-Tid1$_S$ as indicated. Three days later, these cells were fixed and stained with 4′,6-diamidino-2-phenylindole (*blue, top*) and antiactive caspase-3 antibody (*red, bottom*). Red cells were counted as active caspase-3–positive cells; *% active caspase-3 cell*, red cell number per total cell number. *B,* SK-BR-3 cells were treated with viruses as in *A*, followed by incubation with or without the caspase inhibitor (z-Val-Ala-Asp, 25 imol/L). The growth of these treated cells was assessed by MTT assay at the indicated days after infection. The MTT value of control virus–infected cells at day 6 was taken as 100%. *C.* Ad-Tid1$_S$–infected SK-BR-3 cells were coinfected with Ad-EV or Ad-Tid1$_S$ mutants (HQ or CT) as indicated. The growth of these cells was analyzed by MTT assay at the indicated times after infection. The MTT value of day 6 control virus–infected cells was taken as 100%. *D,* SK-BR-3 cells were treated as in *C*. Three days later, the apoptosis of these cells was measured using TUNEL assay; *% apoptosis*, red cell number per total cell number. Results are expressed as the mean ± SE.

infected SK-BR-3 cells were coinfected with individual Ad-Tid1$_S$ mutants. Overexpression of Tid1 with a mutated J domain (HQ) or a Tid1 deletion mutant only expressing COOH-terminal of Tid1 (CT) blocked the apoptosis of Ad-Tid1$_S$–infected SK-BR-3 cells (Fig. 5*C* and *D*). Collectively, these results suggest that increased Tid1 in ErbB-2–overexpressing cells leads to apoptosis, and the Tid1 domains required for ErbB-2 interaction (COOH-terminal) and HSP70 binding (J domain) are critical in this Tid1-mediated PCD of cancer cells.

We next investigated the activity and the protein level of ErbB-2 in SK-BR-3 cells infected with Ad-Tid1$_S$ and found that these cells, compared with cells treated with control virus, had significantly less ErbB-2 and phospho–ErbB-2 proteins but had similar amounts of actin (Fig. 6*A*). Because HSP70 may be involved in regulating ErbB-2 stability by ubiquitination-mediated degradation and because Tid1 is a cochaperone of HSP70 (14, 15), we wondered whether Tid1 has a role in destabilizing ErbB-2 protein by enhancing ErbB-2 ubiquitination. As shown in Fig. 6*B*, increased expression of Tid1$_S$ significantly promoted the ubiquitination of ErbB-2, as revealed by antiubiquitin immunoblot analysis in immunoprecipitates of ErbB-2 from cells infected with Ad-Tid1$_S$ (Fig. 6*B, left*, compare *Lane 3* with *Lanes 1* and *2*). Because ubiquitinated proteins are highly unstable and are quickly degraded by proteasomes, the proteasome inhibitor MG-132 was added to the cells 4 hours before lysing the cells to facilitate the observation of the Tid1-dependent ErbB-2 ubiquitination (Fig. 6*B, right,* compare *Lane 3* with *Lanes 1* and *2*). Because Tid1-CT mutant binds to ErbB-2 signaling domain and thus may serve as a dominant negative by inhibiting the binding of Tid1 to ErbB-2, it also was interesting to test whether the down-regulation of ErbB-2 could be blocked by overexpression of Tid1-CT in SK-BR-3 cells treated with Ad-Tid1$_S$. The ErbB-2 down-regulation in Ad-Tid1$_S$–treated SK-BR3 cells was reversed by coinfecting these cells with Ad-Tid1-CT (Fig. 6*C*). We also observed some increase in ErbB-2 expression in SK-BR3 cells infected with Ad-Tid1-CT alone, which might have resulted from inhibition of endogenous Tid1-mediated down-regulation of ErbB-2 (Fig. 6*C*). Collectively, these data suggest that Tid1 induces ErbB-2 down-regulation via the ubiquitin pathway.

Tyrosine phosphorylation of specific cytoplasmic residues of ErbB-2 recruits binding of signaling molecules, including adaptor molecules that allow activation of specific intracellular oncogenic pathways (2, 3, 16, 17). Among them, mitogen-activated protein kinase (MAPK) pathways and the phosphatidylinositol 3′-kinase/AKT pathway are known to supply proliferative and survival signals, and these pathways have been shown to be critical for the malignant properties of ErbB-2–dependent carcinomas (2, 17, 18). Thus, we investigated whether the activities of these pathways were affected by ErbB-2 protein reduction in Ad-Tid1$_S$–infected SK-BR-3 cells. Phosphorylation of MAPKs, ERK1/2, and big mitogen-activated protein kinase 1 (BMK1)/ERK5 was substantially attenuated in the cells treated with Ad-Tid1$_S$ (Fig. 6*D*). In contrast, there was no difference in AKT phosphorylation between cells infected with Ad-Tid1$_S$ and with control viruses. These results indicated that MAPK pathways, ERK1/2, and BMK1 cascades are the major targeted signaling pathways during Tid1-dependent ErbB-2 deactivation. We next examined the contribution of each of these attenuated MAPK pathways to Ad-Tid1$_S$–induced PCD of carcinoma cells by selectively activating these pathways individually or jointly using MEK1E, the dominant active form of MEK1 (for the ERK1/2 pathway), and/or MEK5D, the dominant active form of MEK5 (for the BMK1 pathway) in Ad-Tid1$_S$–infected SK-BR-3 cells (Fig. 7*A*). We found that the expression of MEK1E or MEK5D up-regulated the activity of ERK1/2 or BMK1, respectively, and partially inhibited the PCD of SK-BR-3 induced by increased Tid1 expression (Fig. 7*B* and *C*). Cotransfection of MEK1E and MEK5D into Ad-Tid1$_S$–infected SK-BR-3 cells had an additive effect in rescuing these cells from apoptosis (Fig. 7*B* and *C*), indicating that although the activity of ERK1/2 and BMK1 pathways is critical for sustaining SK-BR-3 cell growth and survival, there is little overlap between these two pathways in delivering the survival signals from ErbB-2 for SK-BR-3 cells.

We next tested whether increased Tid1 expression inhibited the development of ErbB-2–dependent tumors in animals. A mouse ErbB-2–dependent mammary carcinoma cell line (McNeuA) was

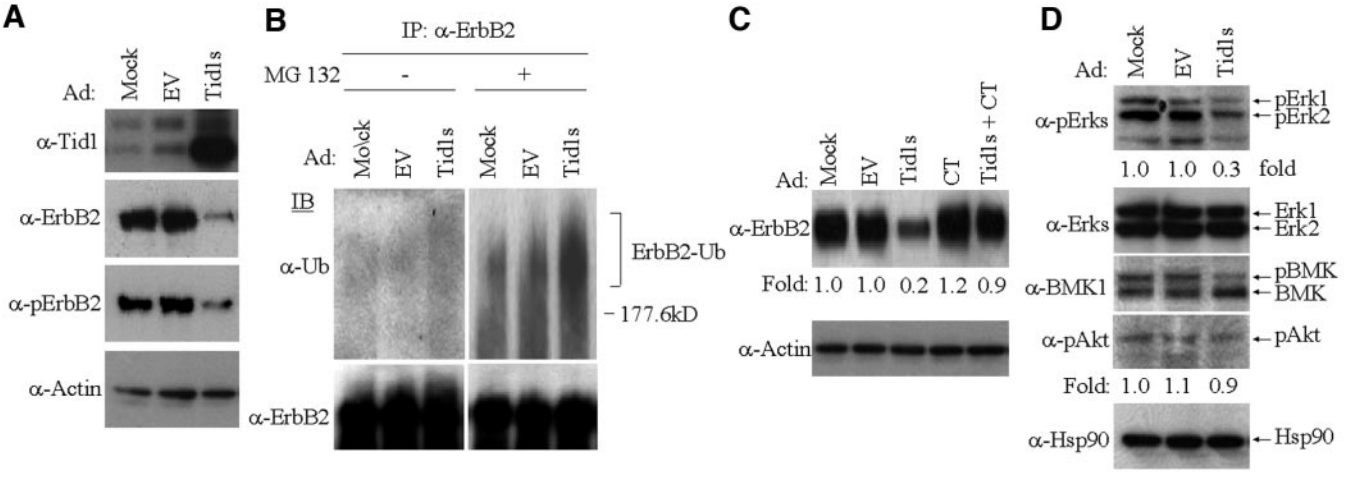

Fig. 6. Tid1$_S$ negatively regulates ErbB-2 signaling pathways by enhancing the degradation of ErbB-2. *A.* SK-BR-3 cells were mock infected or were infected with Ad-EV or Ad-Tid1$_S$ as indicated and were lysed after 2 days. Cell lysates were immunoblotted with the indicated antibodies. *B.* Cells infected with control viruses or Ad-Tid1$_S$ as indicated were treated with (*right*) or without (*left*) 50 μmol/L proteasome inhibitor MG-132 for 4 hours before lysis. The total cell lysates were immunoprecipitated with anti–ErbB-2 antibody, followed by SDS-PAGE and immunoblot with either antiubiquitin (*α-Ub*; *top*) or anti–ErbB-2 antibodies (*bottom*). *C.* SK-BR-3 cells were mock infected (*Lane 1*) or infected with Ad-EV (*Lane 2*), Ad-Tid1$_S$ (*Lane 3*), Ad-Tid1$_S$-CT (*Lane 4*), or with the combination of Ad-Tid1$_S$ and Ad-Tid1$_S$-CT (*Lane 5*) as indicated. Two days later, cell extracts from these infected cells were analyzed by immunoblot analysis with anti–ErbB-2 antibody (*top*) or antiactin antibody (*bottom*). *D.* The same total cell lysates from *B*, without MG-132 treatment, were analyzed by SDS-PAGE, followed by immunoblot anaylsis with anti-pERK1/2, anti-ERK1/2, anti-BMK1, anti-pAkt, or anti-HSP90 antibodies as indicated. Fold of activation of ERK1/2 or Akt was estimated by scanning the immunoblots of the first or third panel using a densitometer. The intensity of band derived from mock-infected cells was taken as one.

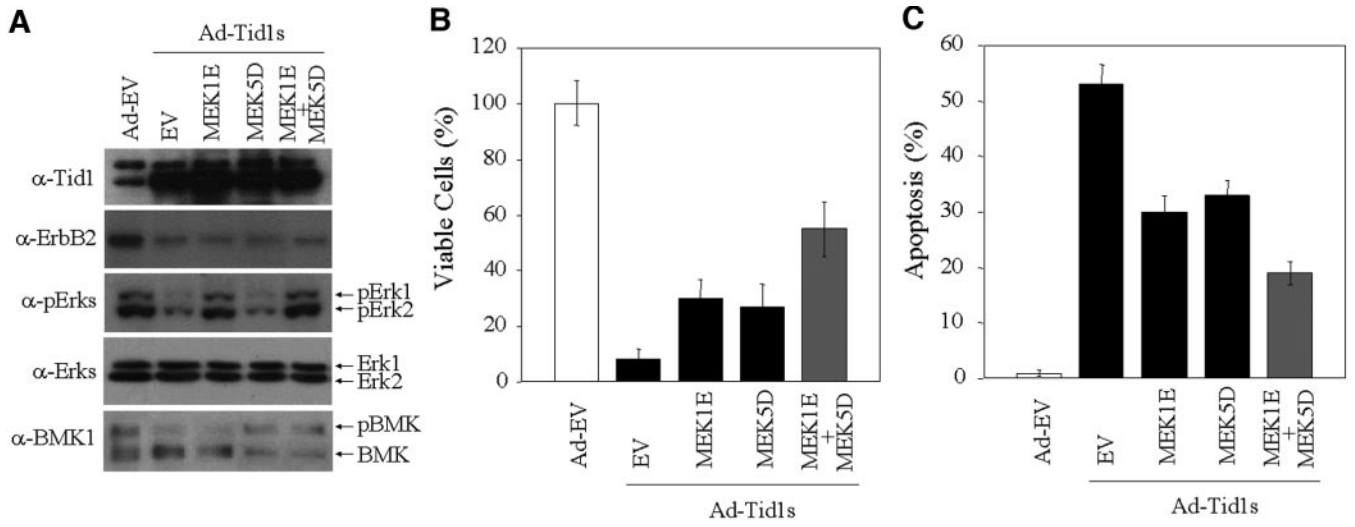

Fig. 7. Down-regulation of ERK1/2 and BMK1 MAPK pathways contributes to the Tid1-mediated PCD of mammary cancer cells overexpressing ErbB-2. *A.* SK-BR-3 cells were incubated with Ad-Tid1$_S$ for 6 hours, and the medium then was replaced with fresh medium. The cells then were transfected with expression plasmids encoding MEK1E and/or MEK5D as indicated. Cell lysates were prepared 2 days after transfection and immunoblotted with the indicated antibodies. *B.* SK-BR-3 cells were infected and later transfected as in *A*. Four days after transfection, the number of viable cells was assessed by MTT assay. The MTT value of Ad-EV–infected cells was taken as 100%. *C,* SK-BR-3 cells were treated as in *A*. Three days after transfection, apoptosis of these cells was measured using TUNEL assay; *% apoptosis,* red cell number per total cell number. Results are expressed as the mean ± SE.

infected with Ad-Tid1$_S$ (Fig. 8*A*), and these treated cells stopped proliferating and started to die 3 days after infection (Fig. 8*B*), similar to what we observed in SK-BR-3 and BT-474 cells. Because murine syngeneic models of this mammary carcinoma are available (7), we investigated whether increased expression of Tid1$_S$ would inhibit the growth of ErbB-2–dependent tumors in mice. McNeuA cells first were infected with Ad-EV or Ad-Tid1$_S$ and then subcutaneously inoculated into FVB/NJ syngeneic mice. Whereas all of the mice injected with noninfected cells or cells infected with Ad-EV developed tumors, none of the mice injected with Ad-Tid1$_S$–infected cells showed progressive tumor growth during the observation period (Fig. 8*C*). To investigate whether administration of Ad-Tid1$_S$ intratumorally would attenuate the growth of pre-existing ErbB-2–dependent tumors, McNeuA cells first were inoculated subcutaneously into the right flank of FVB/NJ syngenic mice. After the tumors developed to ~30 mm^3, the tumor-bearing mice were administered intratumorally with vehicle, control virus, or Ad-Tid1$_S$ on days 8, 12, and 14. The treatment of Ad-Tid1$_S$ induced a significant reduction in tumor volumes: ~70% reduction compared with treatment with vehicle or ~50% reduction compared with Ad-EV (Fig. 8*D*). Collectively, these results suggest that increased expression of Tid1 has an antitumor effect on the development of ErbB-2–dependent carcinomas in animals.

DISCUSSION

Overexpression of the transmembrane receptor tyrosine kinase ErbB-2 is frequent in mammary and ovarian tumors and is indicative of an unfavorable clinical prognosis. Thus, ErbB-2 is known as a molecular target for therapy through reduction of ErbB-2 signaling. One physiologic mechanism to reduce oncogenic signaling of the ErbB family of growth factor receptors is through down-regulation of their expression on the cell surface by ligand-induced endocytosis, followed by lysosome-mediated degradation. Intracellular Cbl proteins interact with ErbB-1 during this process, thereby facilitating its ubiquitination and subsequent degradation (19). However, ErbB-2 fails to bind Cbl and thus avoids ubiquitination and degradation mediated by Cbl proteins. Other ErbB family members that heterodimerize with ErbB-2 also can escape the Cbl-dependent lysosomal degradation, resulting in enhanced

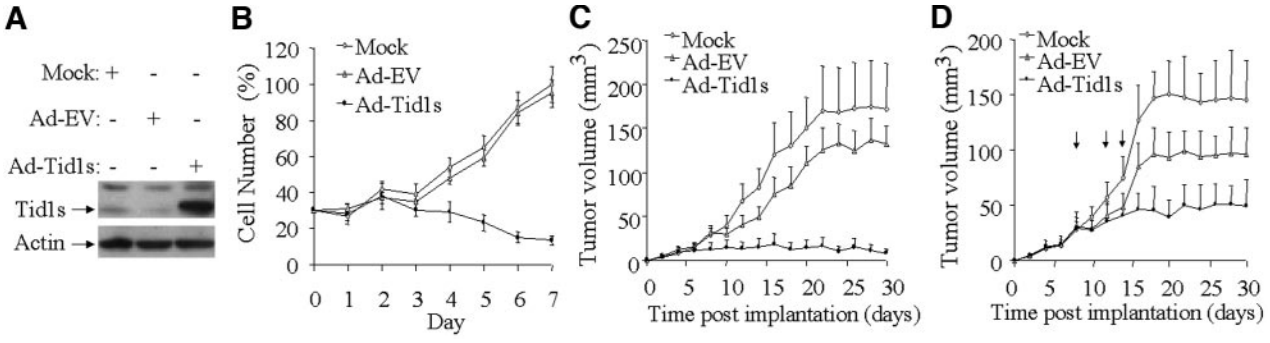

Fig. 8. Increased cellular Tid1 inhibits the growth of ErbB-2–dependent tumors in animals. *A,* increased expression of Tid1$_S$ in McNeuA cells infected with Ad-Tid1$_S$. McNeuA cells were mock infected or infected with Ad-EV or Ad-Tid1$_S$. Two days later, the cells were lysed and analyzed by immunoblot analysis using anti-Tid1 or antiactin antibodies as indicated. *B,* McNeuA cells were mock infected or infected with Ad-EV or Ad-Tid1$_S$. The growth of these cells was examined by MTT assay at the indicated times after infection. The MTT value of mock-infected cells was taken as 100%. *C,* McNeuA cells were treated as in *A*. Two days after treatment, these cells (1×10^6 cells) were inoculated subcutaneously into the right flank of FVB/NJ mice (five mice per group). Tumor volumes were measured at the indicated day after injection. *D,* McNeuA cells (1×10^6 cells) were subcutaneously inoculated into FVB/NJ mice and later allowed to develop tumors to a size of ~30 mm^3 (8 days). These tumor-bearing mice then were randomized into three treatment groups (six mice per group). On days 8, 12, and 14 after implantation, progressively growing McNeuA tumors were treated with intratumoral injections of Ad-Tid1$_S$, Ad-EV, or PBS (vehicle) as indicated. Tumor volumes were measured at the indicated day after initial McNeuA cell injection. Results are expressed as the mean ± SE.

mitogenic signaling. Herein, we found that intracellular Tid1 protein interacts with the signaling domain of ErbB-2 and promotes the ubiquitination and degradation for ErbB-2, leading to subsequent attenuation of the oncogenic signaling from ErbB-2 in carcinoma cells. It has been shown that one mechanism of ErbB-2 ubiquitination and degradation is mediated by an HSP70-dependent pathway in which carboxyl terminus HSP70-interacting protein (CHIP) serves as an ubiquitin ligase (14, 20). However, the mechanism by which ErbB-2 is brought to the HSP70/CHIP complex is poorly understood. Our finding of the interaction of ErbB-2 with Tid1, an HSP70 cochaperone protein, and the subsequent enhancement of ErbB-2 ubiquitination suggests that Tid1 may participate in ErbB-2 degradation mediated by the HSP70/CHIP complex by facilitating the association of ErbB-2 with HSP70 chaperones. Tid1 also contains a cysteine-rich domain, which may serve as a RING finger domain critical for the function of ubiquitin ligases (21). However, whether Tid1 is directly involved in ubiquitination of ErbB-2 is not clear and needs additional investigation.

As shown in Fig. 6*D*, we observed no change in AKT activity after down-regulation of ErbB-2 level by increased expression of Tid1 in SK-Br-3 cells. This is in contrast to several reports that have described a decrease in AKT activity after reduction of ErbB-2 levels in cancer cells (2, 22). In these reports, down-regulation of ErbB-2 was achieved by treatment with Herceptin, ErbB-2–specific antisense oligonucleotides, or ansamycin antibiotics. These treatments, except for ansamycin, are specific for ErbB-2. In our system, however, down-regulation is achieved through Tid1, and Tid1 has been reported to modulate various oncogenic signaling pathways other than ErbB-2, such as Ras, Janus-activated kinase/signal transducers and activators of transcription, and nuclear factor κB pathways (11, 18, 23). Thus, it may be that activation of these pathways by Tid1 compensates for the reduction in oncogenic signals derived from ErbB-2 itself. To this end, AKT has been shown to be either a downstream or an upstream regulator of nuclear factor κB (24). Thus, we suspect that increased expression of Tid1 in SK-Br-3 cells may influence AKT activity indirectly through its regulation of signaling pathway(s), such as nuclear factor κB.

Tid1 is the only human homologue of the *Drosophila* tumor suppressor gene Tid56. Tid56 null mutation causes tumorous imaginal discs resulting from continuous cell proliferation without differentiation (5, 13). To date, the mechanism of tumor suppression of Tid56 in *Drosophila* and the cellular function of Tid1 in human tumorigenesis are poorly understood. In light of our finding that human Tid1 attenuates ErbB-2 activity and blocks the proliferation of ErbB-2–overexpressing cells, it is possible that Tid56 regulates the activity of an epidermal growth factor receptor (EGFR) homologue in *Drosophila*. In the *Drosophila* genome, there is only one member of the EGFR family, DER, which is highly expressed in imaginal discs and has an instructive role in the proliferation and differentiation of these discs (25). DER activity is tightly regulated in time and space by positive and negative regulators to ensure the precise development of the discs. It is likely that the antitumor function of Tid56 results from, at least in part, its ability to negatively regulate DER activity during larval life. Additionally, in Fig. 8, we have shown that Ad-Tid1$_S$ treatment of ErbB-2–dependent tumors resulted in significant reduction in tumor volumes in a syngeneic tumor model. Although there are technical drawbacks to using adenoviruses, such as variations in infectivity of cultured tumor cell lines and variable nonspecific cytotoxic effects, these studies showed that Ad-Tid1$_S$ has significant therapeutic implications for mammalian tumors.

Tid1 also has been shown to regulate the activity of other mitogenic or oncogenic intracellular proteins, such as human papillomavirus E7 oncoprotein, RasGAP, Jak2 kinase, and HTLV-1 Tax protein (11, 18, 23, 26). Most of these molecules are known to be carcinogenic in various human tissues. It is possible that, in addition to ErbB-2, Tid1 also exerts its antitumor function through modulating the activities of these signaling pathways. Most recently, it has been shown that reduction of physiologic levels of Tid1 in cancer cell lines resulted in higher resistance to apoptosis induced by multiple exogenous stimuli, including tumor necrosis factor α (27), suggesting that Tid1 could exercise its tumor suppressor activity by playing a role as a cell death regulator in mammalian cells. Therefore, the potential anticancer activity of Tid1 in animals may affect multiple oncogenic pathways. To clearly determine the function of Tid1 in tumor development, it is critical to establish an animal model in which the expression of Tid1 in various cancer prone tissues can be modulated. The global deletion of Tid1 leads to early embryonic lethality in Tid1-null mice, preventing us from investigating the role of Tid1 in tumorigenesis in adult animals (6). Thus, we have generated a conditional knockout mouse model for the *Tid1* gene to circumvent the issue of early death in Tid1-null mice. We currently are deleting the *Tid1* gene in selective tissues (*e.g.,* mammary epithelium) of these mice by crossing them with transgenic mice carrying tissue-specific expressed *Cre* genes to evaluate the contribution of Tid1 toward various aspects of malignancies in different tissues of mammals.

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Tid1 Negatively Regulates the Migratory Potential of Cancer Cells by Inhibiting the Production of Interleukin-8

Sung-Woo Kim,[1] Masaaki Hayashi,[1] Jeng-Fan Lo,[1] Colleen Fearns,[1] Rong Xiang,[1] Gwendal Lazennec,[3] Young Yang,[2] and Jiing-Dwan Lee[1]

[1]Department of Immunology, The Scripps Research Institute, La Jolla; [2]Johnson and Johnson Pharmaceutical Research and Development, San Diego, California; and [3]Unité Institut National de la Sante et de la Recherche Medicale 540, Montpellier, France

Abstract

Tid1 is the human homologue of the *Drosophila* tumor suppressor, Tid56. Reducing the expression of Tid1 in MDA-MB231 breast cancer cells enhanced their migration without affecting their survival or growth rate. From microarray screening, we discovered that after Tid1 depletion, the mRNA level of interleukin-8 (IL-8) was significantly increased in these cancer cells, which consequently increased secretion of IL-8 protein by 3.5-fold. The enhanced migration of these Tid1-knockdown cells was blocked by reducing the IL-8 expression or by adding an IL-8 neutralizing antibody to the culture medium, suggesting that enhancement of cell motility in these Tid1-deficient cells is dependent on the *de novo* synthesis of IL-8. Subsequently, we found that abrogating the nuclear factor κB binding site in the IL-8 promoter completely blocked the Tid1 depletion–induced IL-8 expression in the breast cancer cells. As increased IL-8 levels are known to promote tumor metastasis, we tested the effect of Tid1 knockdown on tumor metastasis and found that Tid1 depletion enhanced the metastasis of breast cancer cells in animals. Together, these results indicate that Tid1 negatively regulates the motility and metastasis of breast cancer cells, most likely through attenuation of nuclear factor κB activity on the promoter of the *IL8* gene. (Cancer Res 2005; 65(19): 8784-91)

Introduction

Tumor metastasis is the major reason for morbidity and death in cancer patients. Metastasis is the process by which tumor cells spread from their site of origin to distant sites after gaining access to the circulatory system. Active cell migration is required for a cancer cell to establish itself at a second site. The migratory capacity of a cell can be regulated by a number of physiologic factors, including growth factors, extracellular matrix components, and cytokines. Therefore, understanding molecular mechanisms modulating the migratory processes of tumor cells should provide important information in developing therapeutic interventions for tumor metastasis.

The *Drosophilal(2)tid* gene, *Tid56*, is the first and only member of the DnaJ cochaperone family that has been classified as a tumor suppressor. The null mutation of the *Tid56* gene not only keeps imaginal discs from differentiating but also leads to lethal tumorigenesis during the early developmental larval stage (1). For Tid1, the only mammalian counterpart of Tid56, it has been reported that increased expression of Tid1 in human lung adenocarcinoma cell lines reduced the potential for colony formation in soft agar (2) and Tid1-depleted U2OS cells showed augmented colony formation in soft agar (3). Recently, Canamasas et al. showed that loss of Tid1 expression was associated with human basal cell carcinoma but not with normal keratinocytes (4). Similarly, Trentin et al. also reported a Tid1 mutation in human glioma cells and introduction of wild-type Tid1 into these cancer cells induced their apoptosis (5). These observations suggest that Tid1 plays a role in human carcinogenesis. However, the molecular mechanisms involved in these phenomena are poorly understood.

As a DnaJ protein, Tid1 serves as a cochaperone and regulatory factor for the Hsp70 family of molecular chaperones, and is characterized by a J-domain, a highly conserved tetrahelical domain that binds to Hsp70s to regulate their activity and provide substrate specificity. Recently, we showed that the cochaperonic and regulatory function of Tid1 on Hsp70 is required for Tid1 to reduce the malignant signals derived from ErbB2/Her2 by promoting ubiquitination and consequent degradation of ErbB2 (6). By this mechanism, increased expression of Tid1 induces apoptosis in ErbB2-overexpressing breast carcinoma cells and inhibits the growth of ErbB2-dependent tumors in animals. However, increased expression of Tid1 has no effect on the uncontrolled proliferation of breast carcinoma cells with low expression levels of ErbB2 (MDA-MB231). To investigate the role of Tid1 in these cells, we depleted endogenous Tid1 in these cancer cells using Tid1-specific short interfering RNA (siRNA) oligos and found that the migratory potential of these cells was significantly increased. Using microarray screening, we found that reducing Tid1 protein in MDA-MB231 cells up-regulated the *de novo* synthesis of interleukin-8 (IL-8), which is known to modulate cancer cell migration and metastasis. Blocking the production of IL-8 or neutralizing the activity of IL-8 in these Tid1-depleted MDA-MB231 cells almost completely abolished the increase in their motility induced by Tid1 depletion, indicating that Tid1 depletion–induced enhancement of cell migration was caused by increased production of IL-8. Induction of IL-8 expression is also known to be the reason for the promotion of breast cancer cell migration stimulated by the tissue factor-factor VIIa (FVIIa) pathway (7). We investigated whether Tid1 has a role in regulating FVIIa-mediated IL-8 production and consequent up-regulation of cancer cell migration, and found that increased expression of Tid1 in breast carcinoma cells effectively blocked both FVIIa-induced IL-8 synthesis and the resulting enhancement of cell migration. Because increased IL-8 levels are associated with increased metastasis of tumor cells (8, 9), we examined whether Tid1 knockdown could promote tumor metastasis and found that depletion of cellular Tid1 in breast carcinoma cells enhanced their metastatic capability.

Requests for reprints: Jiing-Dwan Lee, Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. Phone: 858-784-8703; Fax: 858-784-8343; E-mail: jdlee@scripps.edu.

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Materials and Methods

Reagent and antibodies. FVIIa was purchased from Enzyme Research Laboratories (South Bend, IN) and thrombin was kindly provided by Dr. W. Ruf at The Scripps Research Institute (La Jolla, CA). Antibodies against β-actin (C-11) and nuclear factor κB (NF-κB) p-65 (SC-109) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-human IL-8 neutralization antibody and ELISA kit for detecting IL-8 production were purchased from R&D Systems (Minneapolis, MN). Anti-Tid1 antibody was kindly provided by Dr. Maria Rozakis-Adcok (McMaster University, Hamilton, Ontario, Canada).

RNAi and transfection. RNAi-mediated gene knockdown was done with the 19-nucleotide targets using siRNAs as follows:

siTid1-1 sense, 5′-GGAGUUCACCGUGAACAUCdTdT-3′
siTid1-1 antisense, 5′-GAUGUUCACGGUGAACUCCdTdT-3′
siTid1-5 sense, 5′-CAGCUACGGCUACGGAGACdTdT-3′
siTid1-5 antisense, 5′-GUCUCCGUAGCCGUAGCUGdTdT-3′
IL-8 sense, 5′-ACCACCGGAAGGAACCAUCdTdT-3′
IL-8 antisense, 5′-GAUGGUUCCUUCCGGUGGUdTdT-3′
siControl sense, 5′-UUCUCCGAACGUGUCACGUdTdT-3′
siControl antisense, 5′-ACGUGACACGUUCGGAGAAdTdT-3′

MDA-MB231 (2×10^5) cells were transfected with a final concentration of 100 nmol/L siRNAs using GenePORTER I (San Diego, CA) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were split, incubated for 8 hours with DMEM containing 10% fetal bovine serum, and then starved in DMEM containing 0.1% fetal bovine serum overnight for the following migration assays.

Recombinant adenoviruses and infections. A full-length cDNA encoding human $Tid1_S$ and its J-domain mutant (Tid1HQ) were cloned into the *Bgl*II and *Hin*dIII sites of pAdTrack-CMV adenovirus shuttle vector (Q-Biogene, Carlsbad, CA) as described previously (4). Adenoviruses containing $Tid1_S$ was used to infect breast cancer cell lines in 24-well plates (1×10^4/well) for 6 hours. Thereafter, the growth medium was replaced every 2 days. Infection efficiency was checked by green fluorescence at a multiplicity of infection of 5×10^4, which was used to reach a 100% infection rate. For generation of Ad-shTid1, a Tid1-specific sequence containing a hairpin loop (5′-GATCCCCAGCTACGGCTACGGAGACTTCAAGAGAGTCTCCGTAGCCGTAGCTGTTTTTGGAAA-3′) was cloned into pSuper vector (10), then moved to pShuttle vector (Q-Biogene) at *Xba*I and *Hin*dIII site as described previously (4).

Cell growth assay. Twenty-four hours posttransfection, cells were split and plated onto polystyrene six-well plates (5×10^4/well) from Corning (Corning, NY) in normal growth medium. Every 24 hours, cells were collected and counted by hemocytometer.

Migration assay. Serum-starved cells were collected by limited trypsin treatment followed by the addition of soybean trypsin inhibitor as described previously (11). The MilliCell membrane (8 μm pore size, polycarbonate filter, 12 mm diameter) from Millipore (Bedford, MA) was precoated with 1 μg/mL rat-tail collagen (Boehringer Mannheim, Indianapolis, IN) for 2 hours and then were air-dried. Cells (1×10^6/well) were added to the upper chamber containing 300 μL of DMEM with 0.5% bovine serum albumin. The upper chamber was placed into the lower chamber containing 400 μL of DMEM with 0.5% bovine serum albumin. For FVIIa-induced migration assay, FVIIa or other stimulants were added to the upper chamber. These chambers were incubated at 37°C containing 5% CO_2 for 6 hours. Nonmigratory cells on the upper surface of the membrane were removed by swiping with a damp cotton swab. The cells that migrated across the filter were fixed in 37% formaldehyde and 25% glutaraldehyde in PBS and stained with 0.1% crystal violet in PBS for 30 minutes. The number of migratory cells per membrane was measured by light microscopy. Each data point is the average of cells in three random fields. Each determination represents the average of at least three individual wells.

Immunofluorescence. MDA-MB231 cells were plated on poly-D-lysine–coated glass coverslips in 12-well plates from BD BioCoat (Bedford, MA) overnight, fixed and permeabilized the next day as described previously (12). After blocking in 2% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 hour, the cells were incubated with antibody against p65 (Santa Cruz Biotechnology) at 1:200 dilution for 1 hour to detect NF-κB. Subsequently, the cells were incubated with Alexa Fluor 568 goat anti-rabbit (red-orange; Molecular Probes, Inc., Eugene, OR) for 30 minutes at a 1:1,000 dilution. At the same time, nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole (0.1 μg/mL) for 30 minutes. These cells were viewed and photographed with a Zeiss Axiovert S100TV microscope.

ELISA. ELISA assays were done using commercial IL-8 ELISA kits from R&D Systems. Conditioned medium from treated or nontreated MDA-MB231 cells were collected from 12-well plates, aliquoted, and stored at

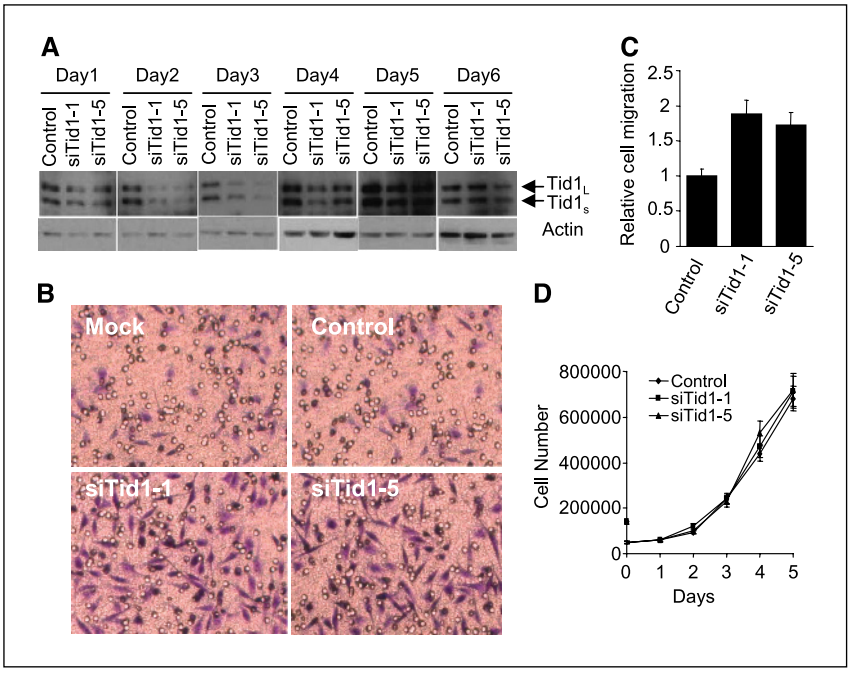
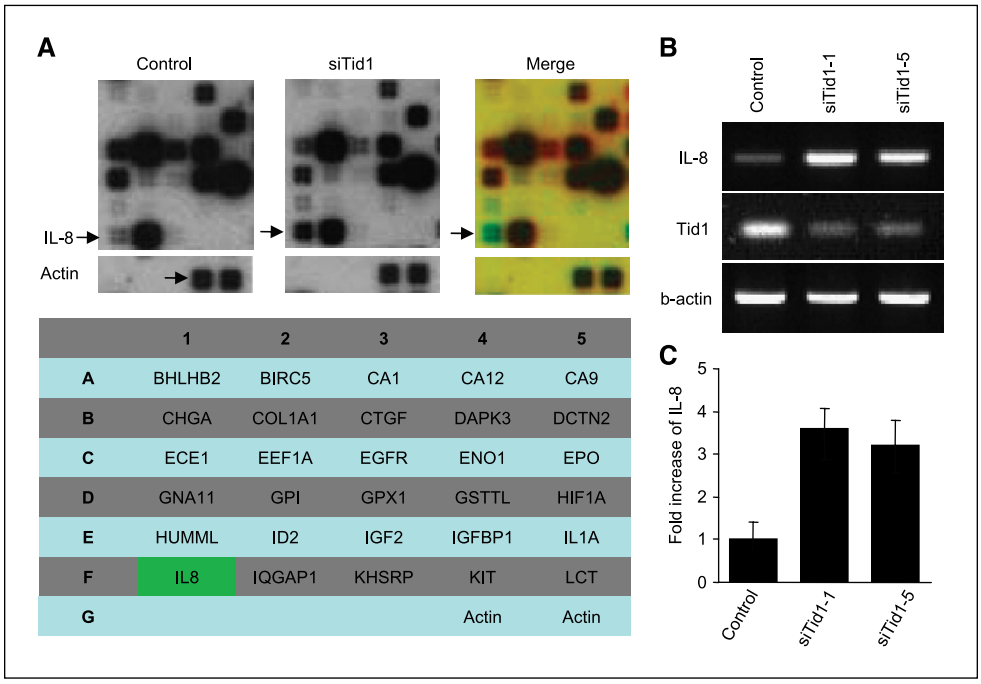


Figure 1. Sequence-specific silencing of *Tid1* gene increases cancer cell motility. MDA-MB231 cells were transfected with the sequence-specific dsRNA oligonucleotides for Tid1 (siTid1-1 or siTid1-5) or control siRNA (negative control). *A,* after transfection, cells were lysed at the indicated day and analyzed by Western blotting using anti-Tid1 antibody. *B,* 1 day after transfection, the subconfluent transfected cells were starved overnight in DMEM containing 0.1% fetal bovine serum and then plated on MilliCell. After 6 hours at 37°C, cells that migrated to the underside of the membrane were stained with crystal violet as described in Materials and Methods. Phase contrast images of migrated cells are shown. *C,* relative cell migration was determined by comparing the number of migratory cells obtained from Tid1-specific dsRNA transfectants to that from cells transfected with control oligos whose value was taken as 1. *D,* MDA-MB231 cells were transfected with control, siTid1-1, or siTid1-5 oligos as described in (*A*). These treated MDA-MB231 cells were grown in normal growth medium and their proliferation was monitored by counting cell numbers at the indicated day using a hemocytometer.



	1	2	3	4	5
A	BHLHB2	BIRC5	CA1	CA12	CA9
B	CHGA	COL1A1	CTGF	DAPK3	DCTN2
C	ECE1	EEF1A	EGFR	ENO1	EPO
D	GNA11	GPI	GPX1	GSTTL	HIF1A
E	HUMML	ID2	IGF2	IGFBP1	IL1A
F	IL8	IQGAP1	KHSRP	KIT	LCT
G				Actin	Actin

Figure 2. Depletion of Tid1 up-regulates IL-8 expression in cancer cells. *A,* MDA-MB231 cells were transfected with Tid1-specific siRNA or control siRNA, and 2 days later, total RNA was isolated and used to produce biotin-labeled cDNA probes. These probes were used separately in hybridization with the microarray membranes (details in Materials and Methods) to analyze the alteration in gene expression profiles in cancer cells after Tid1 knockdown. Each gene in this cDNA array was spotted four times. Actin cDNA (positions G4/5) was spotted as an internal control. Control blot (using probe generated from control cells, *left*) and Tid1-knockdown blot (using probe from Tid1-depleted cells, *middle*) were merged, and green spots indicate the positions of those genes whose expression levels were up-regulated by 2.0-fold or more after Tid1 depletion (*right*). *IL8* gene (position F1) was one of them. *B,* MDA-MB231 cells were transfected with Tid1-specific siRNA or control siRNA, and 2 days later, the expression levels of *IL8* and *Tid1* gene in control or Tid1-depleted cells were examined by semiquantitative reverse transcription-PCR with IL-8 or Tid1-specific primers. *C,* 1 day after transfection with Tid1-specific siRNAs (siTid1-1 or siTid1-5) or control siRNA, MDA-MB231 cells were starved overnight in DMEM containing 0.1% fetal bovine serum. The medium was collected and assayed for IL-8 protein by ELISA. Results are presented as fold increase by taking the value of IL-8 protein in medium of cells transfected with control oligos as one. *Columns,* mean; *bars,* ± SD.

−80°C until assayed. Samples were diluted 5- to 320-fold in deionized water before assaying. Assays were done in triplicate, and readings were compared with standard curves obtained with human recombinant IL-8 provided in the kit.

RNA extraction, reverse transcription-PCR, and cDNA microarray. mRNA was isolated from MDA-MB231 cells using oligo(dT)-conjugated magnetic beads. mRNA (0.5 μg) was used to generate cDNA by reverse transcription using DNA polymerase from Superarray (Frederick, MD). These cDNAs were then used to produce biotin-labeled cDNA probes separately using the Tumor Metastasis Gene Array kit or the Human Hypoxia Signaling Pathway Gene Array kit (Superarray) following the manufacturer's protocol. These arrays were hybridized, washed, and developed according to the manufacturer's instructions. Signal intensities for all genes were quantified and compared using GEArray Analyzer software (Superarray) after normalization to the signals from the housekeeping genes. Genes were identified as up-regulated if the signal from the siTid1-treated cells was >2-fold than that from the siControl-treated cells.

Semiquantitative reverse transcription-PCR assay. MDA-MB231 cells were used to generated mRNA and cDNA as described above. PCR was done using primer pairs specific for Tid1, IL-8, or β-actin:

Tid1 sense: 5′-AAGCAGTACGATGCCTACGG-3′
Tid1 antisense: 5′-TGGCCATCCTCGACTCCTGC-3′
IL-8 sense: 5′-ATGACTTCCAAGCTGGCCGT-3′
IL-8 antisense: 5′-CCTCTTCAAAAACTTCTCCACACC-3′
β-actin sense: 5′-TCCGGAGACGGGGTCA-3′
β-actin antisense: 5′-CCTGCTTGCTGATCCA-3′

The cycling conditions for PCR were as follows: 94°C for 5 minutes, 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; and extension at 72°C for 10 minutes. Ten percent of the PCR products were analyzed on 1.5% agarose gels containing ethidium bromide (0.1 μg/mL). The expression levels of Tid1 or IL-8 were normalized by β-actin expression in the same cells.

Plasmids and reporter gene activity assay. IL-8 promoter corresponding to −1,481/+44 bp was cloned from MDA-MB231 cells and was used for site-directed mutagenesis to mutate activator protein (AP-1), CAAT/enhancer binding protein (C/EBP), and NF-κB sites as described previously. MDA-MB231 cells (1.5×10^5/well) were grown in 12-well plates (Nunc, Naperville, IL) and transiently transfected with 100 ng of the reporter plasmids along with siTid1s or siControl using GenePORTER I. The pRL Renilla luciferase expression vector (10 ng/well) were also cotransfected for normalizing transfection efficiency. The activities of firefly and Renilla luciferase were measured in 20 μL of total cell lysate by using the dual-luciferase reporter assay system (Promega, Madison, WI) following the manufacturer's instructions.

Real-time quantitative reverse transcription-PCR assay. Gene-specific PCR products were continuously measured by means of an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) during 38 cycles. For relative experimental metastasis, specific primers for human hypoxanthine phosphoribosyltransferase (HPRT) message, which do not cross-react with its mouse counterpart were designed (forward, 5′-TTCCTTGGTCAGGCAGTATAATCC-3′; reverse, 5′-AGTCTGGCTTATATCCAACACTTCG-3′). Glyceraldehyde-3-phosphate dehydrogenase was used for normalization.

***In vivo* experimental metastasis studies.** One day after infection, Ad-shTid1 or Ad-Sc-treated MDA-MB-231 breast carcinoma cells (10^6 cells) were injected into the tail vein of CB-17 severe combined immunodeficiency (SCID) mice. Lungs were collected on day 28. Lung tumor burdens were determined by wet lung weights. Afterward, these lungs were fixed or snap-frozen for immunohistochemistry and RNA extraction.

Results

Depletion of Tid1 protein by siRNA increases cancer cell migration. To clarify the function of Tid1 in tumor development, we transfected the breast carcinoma cell line, MDA-MB231, with double-stranded small inhibitory RNA oligonucleotides (siTid1-1 or siTid1-5) designed to specifically silence the *Tid1* gene, which led to significant reduction of the expression levels of both forms of Tid1, $Tid1_L$, and $Tid1_S$, to <20% of control cells (Fig. 1*A* and *B*). Maximal reduction of Tid1 was reached 2 to 3 days after transfection, after which, the expression of Tid1 gradually recovered (Fig. 1*A* and *B*). Next, we determined whether depletion of Tid1 would have any effect on malignant properties such as cell migration and cell growth. We found that migration of siTid1-transfected MDA-MB231 cells was increased 2-fold over that of siControl-transfected cells (Fig. 1*B* and *C*). On the other hand, silencing the *Tid1* gene did not affect the growth of MDA-MB231 cells under normal growing conditions (Fig. 1*D*). These results suggested that Tid1 negatively regulates the motility of MDA-MB231 cells.

Tid1-knockdown enhanced the expression and secretion of IL-8. Cell migration is one of the critical factors for tumor progression and metastasis and a number of physiologic factors could modulate the ability of the cell to migrate (13). To analyze the molecular mechanism responsible for enhanced cell motility induced by Tid1 depletion, microarray-based assays were used to examine the alteration in gene expression profiles in MDA-MB231 cells after suppressing the expression of *Tid1* gene (Fig. 2*A*). We analyzed this microarray data by searching for tumor progression and metastasis-associated genes whose expression levels were altered >2-fold after Tid1 depletion. We found that the message of the *IL8* gene, a common tumor progression and metastasis-associated gene (9, 14), was significantly up-regulated in Tid1-depleded MDA-MB231 cells (Fig. 2*A*). Using semiquantitative reverse transcription-PCR, we further confirmed that the level of IL-8 mRNA was elevated in the cells transfected with siTid1-1 or siTid1-5 compared with that in the cells transfected with siControl (Fig. 2*B*). Moreover, using ELISA, the concentration of IL-8 protein secreted into the medium from the Tid1-suppressed cells was 3- to 4-fold higher than that of control cells (Fig. 2*C*), which concurred with the increased mRNA levels in these siTid1-transfected cells. These results indicated that Tid1 negatively regulates the *de novo* synthesis of IL-8 in breast cancer cells.

The increased motility of Tid1-depleted cells is caused by the increase in IL-8 production. IL-8-induced cell motility has been implicated in the metastatic phenotype of breast cancer in several studies (8, 9, 15, 16). We wondered whether the increased IL-8 production by Tid1-knockdown cells is the reason for the enhanced migration of MDA-MB2321 cancer cells. To test this, we suppressed IL-8 production or neutralized the activity of secreted IL-8 in Tid1-depleted MDA231 and later examined their migratory potential. IL-8 production in Tid1-depleted cells was blocked by cotransfecting siIL-8 and siTid1 oligos into MDA-MB231 cells (Fig. 3*A*), which led to the suppression of both the mRNA and protein level of IL-8 in the Tid1-depleted cells (Fig. 3*A* and *B*) and also reduced the cell motility of Tid1-depleted cells to that of control cells (Fig. 3*C*). The activity of IL-8 secreted by Tid1-depleted cells was inhibited by adding a neutralizing antibody against IL-8 to the culture medium, which led to the reversal of the enhancement of MDA-MB231 cell motility induced by Tid1 depletion (Fig. 3*D*). These data suggested that the increased production of IL-8 by Tid1 depletion causes the motility augmentation in breast cancer cells.

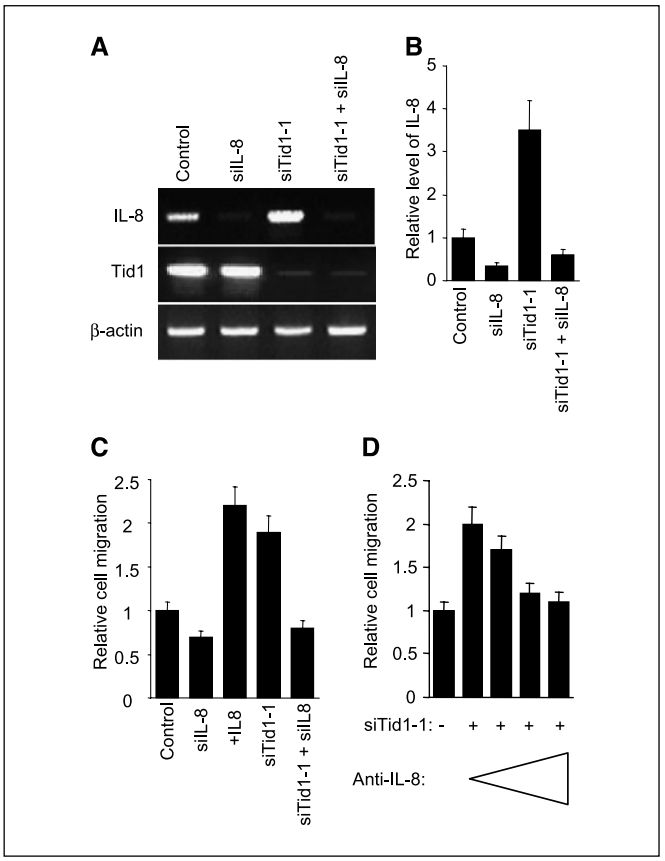


Figure 3. Increased IL-8 production leads to the up-regulation of cell motility in Tid1-depleted cancer cells. *A,* MDA-MB231 cells were transfected with siTid1-1 and/or siIL-8 oligos as indicated to silence the expression of *Tid1* and/or *IL8* genes, respectively. Two days after transfection, the mRNA levels of *Tid1* or *IL8* gene in these cells were analyzed by semiquantitative reverse transcription-PCR with Tid1- or IL-8-specific primers. *B,* MDA-MB231 cells were transfected as described in (*A*). One day after transfection, these cells were starved overnight and later their media was analyzed for IL-8 secretion by ELISA. *C,* the starved cells from (*B*) were used to examine their motility as described in Fig. 1*D*. IL-8 (1 ng/mL) was added to the upper chamber for the indicated experiments. The relative cell migration in these cells was normalized using cells transfected with control oligos whose value was taken as 1. *D,* MDA-MB231 cells were transfected as described in (*A*). Later, these cells were placed in a MilliCell still in the presence of the increasing concentrations of the neutralizing antibody and the motility of these cells was assessed as described in Fig. 1*D*.

Tid1 negatively modulates *de novo* synthesis of IL-8 through regulating NF-κB activity. For Tid1-depleted cells, the increase in IL-8 protein secretion is in accord with the increase in the steady-state level of IL-8 mRNA (Fig. 3*A* and *B*). Several studies showed that the steady-state level of IL-8 mRNA can be dynamically regulated by many factors at the transcriptional level (17). The responsive DNA sequence for IL-8 induction is located in the IL-8 promoter between −133 and −85, and has been shown to contain the binding sites for transcriptional factors, AP-1, NF-κB, and C/EBP1 (18). It has been shown that NF-κB, AP-1, and to a lesser extent, C/EBP binding sites play a crucial role in controlling IL-8 promoter activity (19, 20). These transcriptional factor binding sites in the IL-8 promoter have been individually or jointly mutated to keep them from binding their corresponding transcriptional factors, and the resultant mutated IL-8 promoters were placed separately in front of the luciferase reporter gene. These reporter constructs were individually transfected into Tid1-depleted cells to investigate the responsible *cis*-elements within the IL-8 promoter

for Tid1 depletion–induced IL-8 production (Fig. 4). Consistent with other studies (19, 20), we found that the transcriptional activity of the mutated promoters was significantly reduced compared with the wild-type construct whether transfected with Tid1 siRNA oligos or not (Fig. 4). Mutations within AP-1, C/EBP, and NF-κB binding sites decreased IL-8 promoter activity by about 50%, 30%, and 75%, respectively. However, only mutation of the NF-κB binding site abolished the Tid1-depletion-dependent up-regulation of IL-8 promoter activity (Fig. 4), indicating that Tid1 regulates the IL-8 promoter by modulating NF-κB activity. Furthermore, combined mutation of both the AP-1 and C/EBP sites reduced the activity of the IL-8 promoter to a level similar to NF-κB mutation alone, suggesting that the AP-1 and C/EBP sites are acting coordinately or are necessary for NF-κB activity. Together, these results suggested that regulation of the transcriptional activity of IL-8 induced by Tid1 suppression is dependent on the NF-κB pathway.

Tid1 inhibits FVIIa-induced NF-κB nuclear translocation and cell motility. FVIIa has been implicated in tumor metastasis, in part by its capacity to increase cell migration through induction of IL-8 secretion (7, 21). Because Tid1 negatively regulated the IL-8 production of cancer cells and their subsequent migration, it was of interest to test whether Tid1 could modulate FVIIa-induced migration of breast carcinoma cells. To test this, we increased the expression of cellular $Tid1_S$ in MDA-MB231 cells by infecting them with recombinant adenovirus encoding $Tid1_S$ (Ad-$Tid1_S$). A J-domain mutant of Tid1 (Ad-Tid1HQ) was used in this experiment to examine the involvement of the cochaperonic activity of Tid1 in this FVIIa-mediated cell activation; an empty virus, Ad-EV, was used as a control in these experiments. These cells were starved, treated with FVIIa or thrombin, and later analyzed for IL-8 secretion by ELISA (Fig. 5*B*). Consistent with previous reports (7), IL-8 secretion was increased by treatment with 10 nmol/L FVIIa, a concentration equivalent to that in plasma and IL-8 production was further increased by treatment with 50 nmol/L FVIIa, whereas thrombin has no effect on IL-8 secretion. As expected, increased expression of Tid1 in cancer cells significantly inhibited IL-8 secretion induced by FVIIa. In contrast, infection with Ad-Tid1HQ had no effect on the induction of IL-8 secretion by FVIIa, suggesting that the cochaperonic activity of Tid1 is required for blocking the FVIIa-induced IL-8 production in these cells. Next, we tested whether Tid1 can negatively regulate FVIIa-induced tumor cell motility. The migration of MDA-MB231 cells infected with Ad-$Tid1_S$, Ad-Tid1HQ, or Ad-EV was examined in a MilliCell with or without the presence of FVIIa in the top well. The higher level of cellular $Tid1_S$ (but not Tid1HQ) markedly blocked cell migration induced by FVIIa, which coincides with the ability of $Tid1_S$ (but not Tid1HQ) to block FVIIa-induced IL-8 production in MDA-MB231 cells. Because NF-κB is the dominant pathway for the induction of IL-8 by FVIIa (20) and because Tid1 negatively regulates NF-κB activity (Fig. 4), it is possible that Tid1 blocked FVIIa-induced IL-8 production by inhibiting NF-κB activity. In unstimulated cells, the NF-κB complex was located mainly in the cytoplasm and after FVIIa treatment, a portion of the NF-κB complex translocated into the nucleus (Fig. 5*D*). Increased cellular $Tid1_S$ significantly inhibited this FVIIa-induced NF-κB nuclear translocation in MDA-MB231 cells, as shown in Fig. 5*D*, indicating that Tid1 inhibits FVII-induced IL-8 production and cell migration by blocking NF-κB translocation to the nucleus. Together, these results suggested that Tid1 negatively regulates FVIIa-induced IL-8 expression and the consequent enhancement of cell motility of breast carcinoma cells by blocking activation of the NF-κB

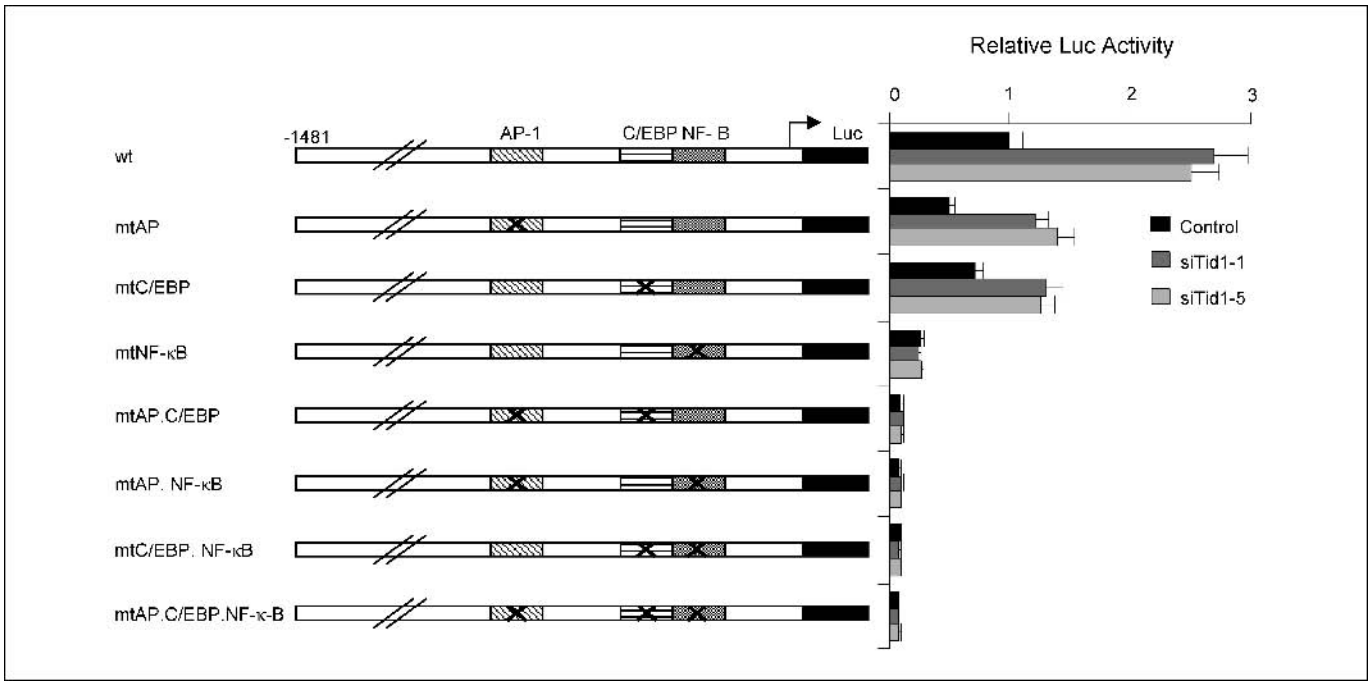


Figure 4. Tid1 depletion–induced *de novo* synthesis of *IL8* gene is dependent on NF-κB activity. *A,* MDA-MB231 cells were separately transfected with reporter plasmids which encode a luciferase gene driven by wild-type, or mutated IL-8 promoters harboring single, double, or triple mutations of AP-1, C/EBP, or NF-κB sites, along with siTid1 or siControl oligos as indicated. The pRL Renilla luciferase expression vector were also transfected into these cells for normalizing transfection efficiency. The cells were then starved overnight and assayed for luciferase activity. The activities of firefly and Renilla luciferase were quantified in the same sample using the dual-luciferase reporter assay (Promega Corporation). The luciferase activities were normalized against cells transfected with the control oligos and a reporter plasmid with the wild-type IL-8 promoter, whose value was taken as 1.

transcriptional factor. Interestingly, the fact that a functional J-domain in Tid1 is critical for this phenomenon indicates that the cochaperonic function of Tid1 to Hsp70 is required for Tid1-mediated negative regulation of NF-κB activation.

Tid1-depletion enhanced metastasis of mammary carcinoma cells. Because IL-8 has been strongly correlated with a metastatic phenotype (8, 9, 15, 16) and Tid1 depletion increased production of IL-8 in MDA-MB231, we tested whether reducing the cellular level of Tid1 can increase the metastatic potential of breast carcinoma cells. We first generated recombinant adenovirus producing shRNA specific for Tid1, Ad-shTid1, to prolong the period of Tid1-depletion in MDA-MB231 cells. The depletion of endogenous Tid1 in Ad-shTid1-infected MDA-MB231 cells can be maintained for >2 weeks (Fig. 6*A*). MDA-MB-231 cells infected with adenovirus- Tid1 shRNA or scramble (Sc) were injected into the tail vein of SCID mice. Four weeks after injection, lung metastases were visualized (Fig. 6*B*) and quantified by wet lung weight (Fig. 6*C*). We found that lung metastasis is significantly higher (~40%) in mice injected with Tid1-depleted cancer cells than in mice injected with cancer cells containing normal levels of Tid1. In addition, the individual tumor burden per lung was measured by quantitative reverse transcription-PCR using primers specific for the human housekeeping gene, *HPRT*, which detects human but not mouse HPRT mRNA (Fig. 6*D*). The expression level of human HPRT in lungs infiltrated with Tid1-depleted tumor cells are ~40% to 50% higher than that in lungs infiltrated with control cells (Fig. 6*D*). These data suggest that Tid1-depletion significantly increase the metastasis rate of breast carcinoma cells.

Discussion

The core IL-8 promoter contains NF-κB, AP-1, and C/EBP-binding sites. Our results showed that transcriptional activation of the IL-8 promoter induced by Tid1-knockdown is mostly dependent on the NF-κB pathway (Fig. 4). FVIIa-induced NF-κB nuclear translocation was also blocked by increased cellular Tid1

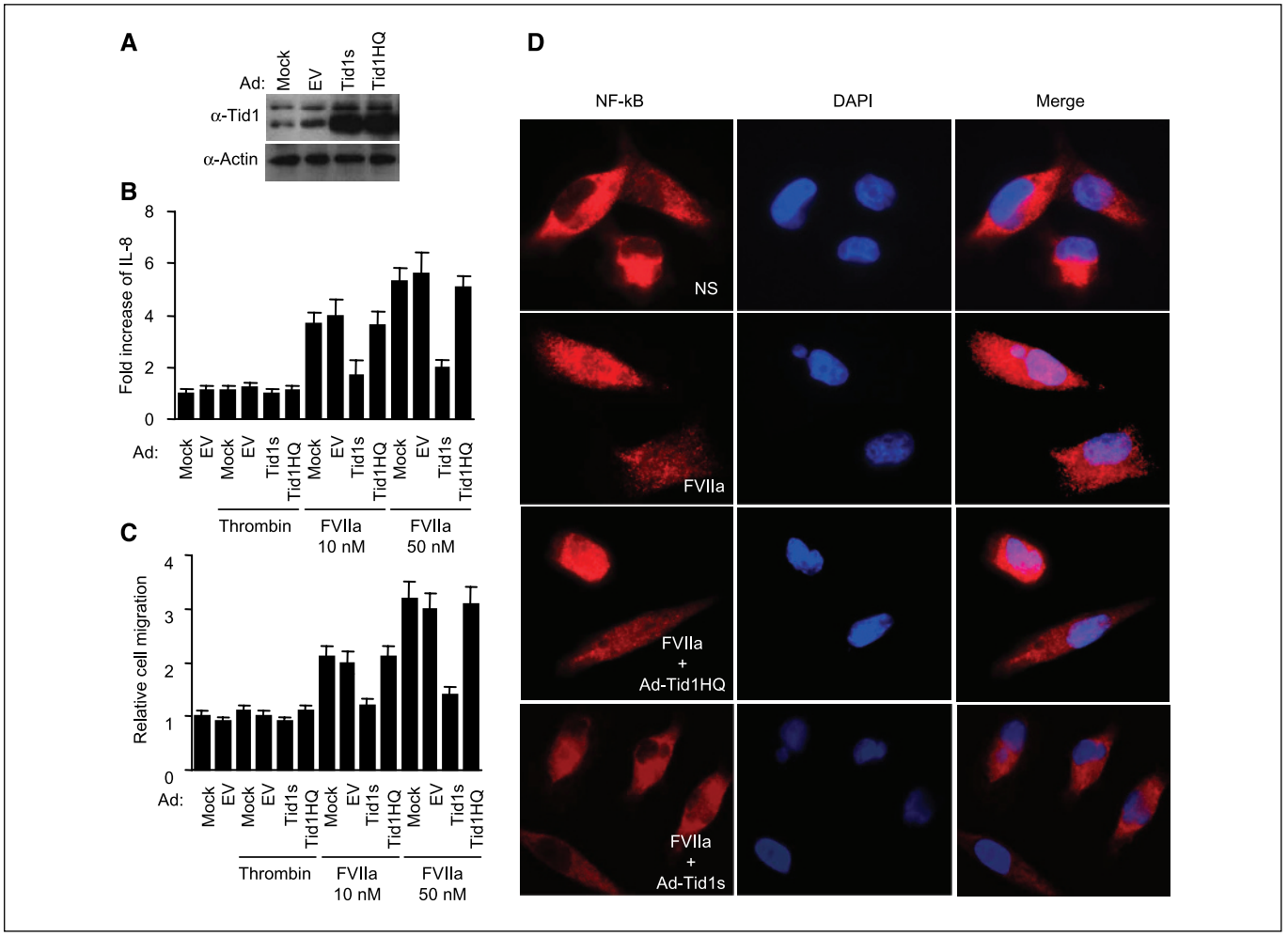


Figure 5. Tid1 inhibits FVIIa-induced IL-8 production and consequent up-regulation of cell motility of breast cancer cells. *A,* MDA-MB231 cells were infected with recombinant adenoviruses encoding $Tid1_S$ (Ad-$Tid1_S$), a J-domain mutant of $Tid1_S$ (Ad-Tid1HQ) or empty virus (Ad-EV). Two days after infection, cells were lysed and analyzed for Tid1 expression by an anti-Tid1 antibody in an immunoblot assay. *B,* MDA-MB231 cells were infected with recombinant adenoviruses as described in (*A*). After 24 hours, these cells were starved overnight and then treated with different concentrations of FVIIa (10 and 50 nmol/L) or thrombin (10 nmol/L) as indicated for 16 hours in 0.1% fetal bovine serum. IL-8 secretion by these cells was then evaluated by ELISA as described in Fig. 2*C*. *C,* the infected starved cells in (*B*) were placed in a MilliCell, and simultaneously, FVII (10 or 50 nmol/L) or thrombin (10 nmol/L) was added to the top chamber of the MilliCell for 6 hours at 37°C. The number of cells that migrated to the underside of the membrane was determined as described in Fig. 1*D*. *D,* the infected quiescent cells in (*B*) were stimulated with or without 50 mmol/L FVIIa for 2 hours at 37°C. Cells were then immunostained with an anti-p65 antibody (Santa Cruz Biotechnology) to detect the localization of NF-κB complex (*left*). Nuclei were visualized using 4′,6-diamidino-2-phenylindole (*middle*). Merged red and blue fluorescence (*right*).

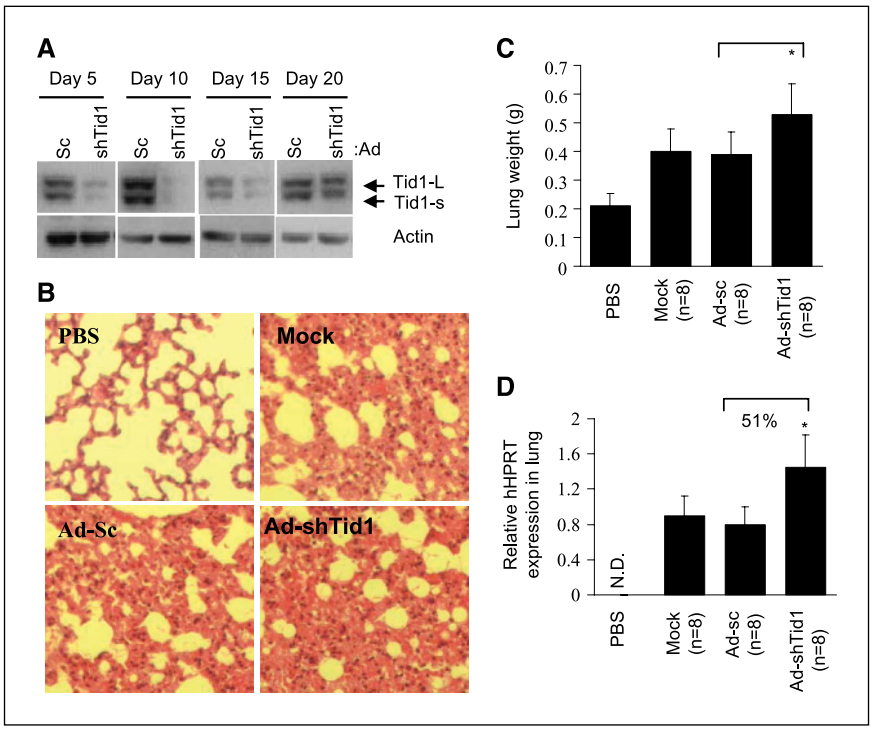


Figure 6. Tid1-depletion increases metastasis of breast carcinoma cells. *A,* MDA-MB231 cells were infected with recombinant adenoviruses encoding shTid1$_S$ (Ad-shTid1$_S$) or with Ad-scramble (Sc) as control. At the indicated day after infection, cells were lysed and analyzed for Tid1 expression by immunoblotting using an anti-Tid1 antibody. One day after infection, cells (1×10^6) infected with Ad-shTid1s, Ad-Sc, or nothing (mock) were collected and injected into the tail vain of CB17-SCID mice. *B,* lungs from these mice were isolated 4 weeks after injection and stained with H&E to check for tumor metastases. *C,* wet lung weights of mice. I.v. injection of MDA-MB231 cells infected with Ad-shTid1, Ad-sc, or nothing (mock). *D,* quantitation of metastases in the abovementioned lungs by quantitative reverse transcription-PCR analysis for human hHPRT mRNA. N.D., not detectable. *Columns,* mean; *bars,* ± SD. Percentages indicate relative enhancement compared with control (Ad-Sc treated) cells. *, $P < 0.05$, Student's *t* test.

(Fig. 5*E*). However, the precise NF-κB regulatory mechanism of Tid1 in breast cancer cells is not clear. In general, NF-κB is sequestered in the cytoplasm in an inactive complex with IκB proteins (22). NF-κB activation is initiated through phosphorylation of IκB by IκB kinases (IKKα/β and IKKγ/Nemo) followed by consequent degradation of the phosphorylated IκB, resulting in nuclear translocation of NF-κB (23, 24). Interestingly, it has been previously shown that Tid1 suppresses tumor necrosis factor-α and Bcl10-induced NF-κB activation by inhibiting IKKβ kinase activity (25). Furthermore, Hsp70, the protein in complex with Tid1, is known to suppress NF-κB activity by binding IKKγ (26, 27). In fact, interaction between Tid1 and Hsp70 was enhanced in the presence of ATP, implicating Tid1 as a regulatory cofactor of Hsp70 (28). Therefore, it is very likely that Tid1, in conjunction with Hsp70, negatively regulates NF-κB activity through modulating IKK activity, and it will be of interest to verify this hypothesis.

The role of IL-8 was observed in several studies in which its expression was strongly correlated with a metastatic phenotype. In breast cancer, IL-8 overexpression has been detected in tumors with highly invasive potential (29), and the expression of IL-8 has also been correlated with metastasis of breast cancer cells to lung after implantation in the mammary fat pad (8). Moreover, a highly metastatic breast cancer line was isolated in which IL-8 expression was significantly up-regulated compared with that of its less metastatic parental line (9). Increased IL-8 expression has also been shown to render nonmetastatic human melanoma cells metastatic (30). In clinical studies, IL-8 was overexpressed in breast tumor tissues compared with that of normal tissues (31). Moreover, the role of IL-8 in tumor development is not only in metastasis but also in promoting tumor cell proliferation and tumor-associated angiogenesis (32). As IL-8 is implicated in various aspects of tumor development and because Tid1 negatively regulates the *de novo* synthesis of IL-8 in tumor cells, it is reasonable to assume that in addition to Tid1's inhibitory effect on IL-8-induced cell migration, Tid1 may also block IL-8-mediated tumor cell growth, invasion, and metastasis, which are currently under investigation.

In Fig. 5, we showed that Tid1 inhibited FVIIa-induced IL-8 production and consequent cell migration of breast tumor cells. Previously, it has been shown that the tissue factor-FVIIa pathway regulated IL-8 expression (20) and that the tissue factor-FVIIa complex promoted melanoma metastasis, which is independent from its role in blood coagulation (33, 34). In breast cancer cells, treatment with an IL-8 neutralizing antibody blocked the cell migration induced by FVIIa, suggesting that the tissue factor-FVIIa pathway modulates the migratory potential of cancer cells through IL-8 production (7). As Tid1 blocks the IL-8 production of cancer cells and the consequent cell migration induced by FVIIa, it is very likely that Tid1 has a role in deterring tumor metastasis promoted by tissue factor-FVIIa pathway. Most recently, the tissue factor-FVIIa protease complex, independent of triggering coagulation, was shown to promote tumor-associated angiogenesis through protease-activated receptor-2 signaling in animals (11). Because NF-κB and the downstream effectors regulated by it have been linked to angiogenesis, and because Tid1 negatively regulates NF-κB activity, it is tempting to investigate whether Tid1 can attenuate the FVIIa-induced development of tumor vasculature.

In summary, we showed that tumor suppressor Tid1 negatively regulated cell motility and metastasis of breast carcinoma cells. This Tid1-dependent effect is, most likely, through the inhibitory role of Tid1 on activation of the transcriptional factor NF-κB, which is critical for the *de novo* synthesis of the *IL8* gene.

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